

Vaccinia Virus-Based Multivalent H5N1 Avian Influenza Vaccines Adjuvanted with IL-15 Confer Sterile Cross-Clade Protection in Mice¹

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The potential for a global influenza pandemic remains significant with epidemiologic and ecologic indicators revealing the entrenchment of the highly pathogenic avian influenza A H5N1 in both wild bird populations and domestic poultry flocks in Asia and in many African and European countries. Indisputably, the single most effective public health intervention in mitigating the devastation such a pandemic could unleash is the availability of a safe and effective vaccine that can be rapidly deployed for pre-exposure vaccination of millions of people. We have developed two vaccinia-based influenza vaccines that are molecularly adjuvanted with the immune stimulatory cytokine IL-15. The pentavalent Wyeth/IL-15/5Flu vaccine expresses the hemagglutinin, neuraminidase, and nucleoprotein derived from the H5N1 influenza virus A/Vietnam/1203/2004 and the matrix proteins M1 and M2 from the H5N1 A/CK/Indonesia/PA/2003 virus on the backbone of a currently licensed smallpox vaccine. The bivalent MVA/IL-15/HA/NA vaccine expresses only the H5 hemagglutinin and N1 neuraminidase on the modified vaccinia virus Ankara (MVA) backbone. Both vaccines induced cross-neutralizing Abs and robust cellular immune responses in vaccinated mice and conferred sterile cross-clade protection when challenged with the H5N1 virus of a different clade. In addition to having potential as a universal influenza vaccine, in the event of an impending pandemic the Wyeth/IL-15/5Flu is also readily amenable to bulk production to cover the global population. For those individuals for whom the use of the Wyeth vaccine is contraindicated, our MVA/IL-15/HA/NA offers a substitute or a prevaccine to be used in a mass vaccination campaign similar to the smallpox eradication campaigns of few decades ago. *The Journal of Immunology*, 2009, 182: 3063–3071.

The past century was witness to three influenza pandemics that ravaged humanity. The “Spanish Flu” of 1918, caused by the emergence of the influenza A H1N1 virus, killed according to the most modest estimates >30 million people world wide, followed by “Asian Flu” in 1958 with the appearance of H2N2 that led to another 1 million deaths (1). The last pandemic of the 20th century, in 1968, was caused by influenza A H3N2 and dubbed “Hong Kong Flu,” reportedly killing 800,000 people in just 6 wk (1). The appearance of the highly pathogenic avian influenza (HPAI)³ A H5N1 virus in Hong Kong in 1997 was thought

to be a harbinger of the first influenza pandemic of the 21st century. Since its first detection the H5N1 virus has spread through Asia, reaching the Middle East, Africa, and Europe within a span of 10 years (2). As of October 2008, the H5N1 virus has been responsible for 245 deaths and 387 confirmed cases of human infection with a case fatality rate of >60% (see the World Health Organization report on the cumulative number of confirmed human cases of avian influenza A/(H5N1); www.who.int/csr/disease/avian_influenza/country/en/).

With the looming threat of a global influenza pandemic, there is a general consensus that the most effective public health intervention in curbing the morbidity, mortality, and economic catastrophe associated with such a pandemic is an effective vaccine. Such a vaccine should be efficacious, safe, and amenable to a rapid scale-up in production to ensure its quick deployability should such a pandemic erupt. In April of 2007, the FDA approved the first vaccine (rgA/Vietnam/1203/2004) for human use against the avian influenza virus H5N1; it is manufactured by Aventis-Sanofi-Pasteur (see U.S. Food and Drug Administration consumer update for April 17, 2007; www.fda.gov/consumer/updates/birdflu043007.html). The vaccine is an inactivated, detergent-extracted subvirion formulation and has been added to the U.S. Strategic National Stockpile to be used in the event of a pandemic but not for current commercial use. Despite its approval as an interim vaccine, the poor immunogenicity of this vaccine seriously undermines the potential utility of this vaccine in the face of an impending pandemic. Evidence of potency decay over time has also been observed in the initially stockpiled lots of this subvirion vaccine, further raising more serious concerns.

Thus, an urgent need exists for a better vaccine; a number of potential candidate vaccines have been developed, and some of these candidates are being evaluated in clinical trials (reviewed in

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³ Abbreviations used in this paper: HPAI, highly pathogenic avian influenza; MDCK, Madin-Darby canine kidney; MOI, multiplicity of infection; MVA, modified vaccinia virus Ankara.

Ref. 3). We have taken the view that a multivalent, live, vector-delivered vaccine carrying multiple immunogenic influenza polypeptides that have been shown to be capable of eliciting protective immune responses both in humans and in animal models, especially if combined with an immunostimulatory cytokine, would yield a superior vaccine. With this objective, we generated vaccinia virus-based vaccine candidates that tandemly express either five genes of H5N1 influenza A virus, namely the H5 hemagglutinin gene, the N1 neuraminidase gene, the nucleoprotein NP gene, and genes encoding the two matrix polypeptides M1 and M2 along with the cytokine IL-15 on the backbone of the Wyeth strain of vaccinia, or only the two surface genes, H5 hemagglutinin and N1 neuraminidase, with IL-15 on the modified vaccinia virus Ankara (MVA) backbone. The cytokine IL-15 has emerged as a powerful immune stimulatory cytokine with a wide range of biological activities. It is involved in the activation, proliferation, and differentiation of CD8⁺ T cells and NK cells and in the maintenance of CD8⁺ memory T cells in addition to supporting the survival of mature dendritic cells. It also plays a role in B cell differentiation, maturation, and Ab secretion, thus making IL-15 an ideal candidate for incorporation as a molecular adjuvant in vectored vaccines. Our vaccine candidates in preclinical mouse challenge studies conferred sterile cross-clade protection with 100% efficacy against highly pathogenic influenza A H5N1 viruses, attesting to their excellent efficacy and potential utility as broadly effective pandemic influenza vaccines.

Materials and Methods

Construction of vaccinia-based vaccine candidates

Influenza virus A/Vietnam/1203/2004 was grown in Madin-Darby canine kidney (MDCK) cells, and the viral RNA was extracted by standard procedures using infected culture supernatants (4). From the extracted viral RNA, cDNA was synthesized by standard RT-PCR and the coding segments of the hemagglutinin, neuraminidase, and nucleoprotein were amplified individually by PCR. The 5' primers contained a synthetic early-late vaccinia promoter added before the initiator codon ATG, and the 3' primer contained a vaccinia transcription terminator sequence, TTTTCT, added after the gene-specific translation terminator codon for each of the genes amplified. In the case of the hemagglutinin gene, the H₀ cleavage site sequence was modified to code for the PSIQYR monobasic motif from the original cognate sequence encoding the PQRERRRKKR polybasic motif. Similarly, in the NP gene the codon 257 was changed from ATT to ATC, but the synonymous codon change still codes for isoleucine to eliminate a potential vaccinia transcription terminator sequence. The matrix genes M1 and M2 were synthesized de novo with vaccinia virus early-late promoters 5' to the ATG start codon, and the TTTTCT vaccinia transcription terminator was added 3' to the natural translation stop codon of the respective gene. The coding region DNA sequences were derived from GenBank entry AY651376 for M1 and M2 (A/CK/Indonesia/PA/2003, H5N1). The coding segment of human IL-15 gene with a 5' vaccinia early-late promoter and a 3' TTTTCT transcriptional terminator sequence has been described previously (5). A mutated version of this construct was also created by changing the ATG start codon of *IL-15* gene to TTG such that no bioactive IL-15 was expressed. The Wyeth New York Board of Health strain of vaccinia was obtained from Wyeth Ayerst Laboratories. MVA (an isolate made in 1974 before the bovine spongiform encephalopathy era) was provided by Dr. B. Moss from the National Institute of Allergy and Infectious Diseases (Bethesda, MD). To create recombinant vaccinia viruses, a pTFHA transfer vector with a 1.8-kb DNA fragment encompassing the hemagglutinin gene of vaccinia and the *Escherichia coli gpt* gene was used as described earlier (5). Wyeth recombinant viruses with five influenza genes (H5 hemagglutinin, N1 neuraminidase, NP, M1, and M2) along with IL-15 were created by first cloning all six genes as a head-to-tail concatamer into the pTFHA transfer vector by standard cloning techniques. A similar three-gene concatamer with H5 hemagglutinin, N1 neuraminidase, and IL-15 was cloned into pTFHA and used to create MVA recombinant viruses. Recombinant viruses were generated by standard procedures as described previously (5) by transfecting the relevant transfer plasmid into Wyeth- or MVA-infected cells and selecting plaques that were resistant to mycophenolic acid. The Wyeth strain of vaccinia and its recombinant derivatives were grown and titered in a CV-1 monkey kidney

cell line from American Type Culture Collection (ATCC), whereas the MVA strain and its recombinant derivatives were grown in a BHK-21 cell line from ATCC.

Western blot analysis

Wyeth recombinant vaccinia viruses were grown in CV-1 monkey kidney cells and the MVA recombinants were grown in BHK-21 cells. When infected cells displayed 75% cytopathic effects, infected monolayers were harvested and cell pellets were resuspended in radioimmune precipitation assay buffer with protease inhibitors to yield a final protein concentration of 10 mg/ml. Infected cell lysates were subjected to SDS-PAGE (10% acrylamide gels) and the separated proteins were transferred to polyvinylidene difluoride membranes for immunoblotting. The following primary Abs were purchased from Abcam and used for the detection of influenza Ags: polyclonal rabbit Ab for N1 neuraminidase (catalog no. 21304-100); polyclonal rabbit Ab for hemagglutinin (catalog no. 21297-100); polyclonal rabbit Ab for nuclear protein NP (catalog no. 21008-100); and polyclonal goat Ab for M1 (catalog no. 21008-100). These Abs were used at a final concentration of 1 µg/ml.

Immunofluorescence

For detection of M2 expression, CV-1 cells were grown in Lab-Tek borosilicate chamber slides and infected with Wyeth recombinants at a multiplicity of infection (MOI) of 0.1 for 24 h. Infected cells were fixed in ethanol/acetone and then reacted with a rabbit polyclonal Ab specific for the M2 protein of H5N1 virus (catalog no. 4333) from ProSci. The detection Ab was a rhodamine-conjugated anti-rabbit Ab.

Detection of IL-15 activity

CV-1 cells were infected with vaccinia virus at a MOI of 10 and infected cells were cultured for 3 days before harvesting the supernatants for IL-15 activity. Harvested supernatants were irradiated (3000 rad) to eliminate infectivity and then tested for IL-15 activity using a commercial ELISA kit for human IL-15 (R&D Systems). Bioactivity was determined by the ability of supernatants to support the growth of an IL-2/IL-15-dependent NK-92 cell line as reported previously (6). After the addition of test supernatants, cells were incubated for 48 h and then pulsed with 1 µCi/ml [³H]thymidine for an additional 6 h. Triplicate samples were counted by scintillation. Cells incubated with medium alone served as a negative control. The proliferative index was calculated as the fold increase in [³H]thymidine uptake above the medium control.

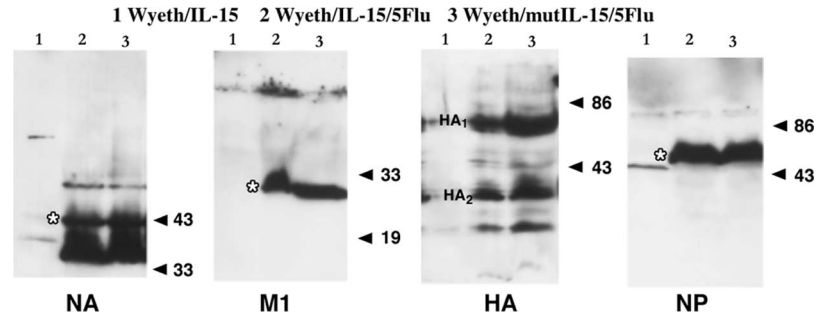
Mice and immunizations

Specific pathogen-free female BALB/c mice (6–10 wk old) were used for immunization studies. All animal procedures were conducted under institutionally approved protocols. Mice were immunized s.c. at the base of the tail with 1 × 10⁷ PFU of vaccinia virus in a volume of 100 µl. A second booster dose was given 4 wk later. Each experimental group consisted of six animals. For comparative analysis, in certain experiments mice were given a single dose of FDA-approved Aventis vaccine (rgA/Vietnam/1203/2004) i.m. (100 µl containing 3 µg of vaccine per mouse injected to the gluteal region).

Assays for immune responses

An ELISA was used to measure the levels of influenza Ag-binding Abs in sera collected from vaccinated mice essentially as described previously except for the Ag preparations used for coating ELISA plates (7). For coating ELISA plates, three different preparations of influenza Ags were used depending on the experiment. For certain experiments, purified baculovirus-expressed recombinant H5 hemagglutinin was used at a concentration of 1 µg/ml from Protean Sciences (catalog no. 3006). We also used H1N1-infected MDCK cell culture supernatants having a 1 × 10⁶ PFU/ml virus titer after 7-fold dilution in carbonate coating buffer. H3N2 A/Aichi/2/68 infected allantoic fluid was also used. In some experiments we used a monovalent subvirion vaccine (rgA/Vietnam/1203/2004) manufactured by Aventis Pasteur with a 30 µg/ml standardized hemagglutinin content after 20-fold dilution in carbonate coating buffer. Neutralizing Ab titers were determined by a microneutralization assay using MDCK cell monolayers with a 100 50% tissue culture-infective dose of the appropriate wild-type H5N1 virus, and the reciprocal of the highest dilution of the serum that completely neutralized this infectivity was taken as the titer. For detecting cellular responses against influenza Ags, an in vitro coculture assay was used. Splenocytes from three animals within a group were pooled, and CD4⁺ and CD8⁺ T lymphocytes were purified negatively using CD4⁺ or CD8⁺ T cell isolation kits from Miltenyi Biotec according to manufacturer's instructions. Purified cells were plated at 2 × 10⁶ cells per well in

FIGURE 1. The expression of integrated H5N1 genes by recombinant Wyeth vaccinia viruses. Recombinant vaccinia infected CV-1 cell lysates were subjected to SDS-PAGE followed by immunoblotting with Abs specific for neuraminidase (NA), matrix protein M1, hemagglutinin (HA), and nucleoprotein NP proteins of influenza A virus. On the right side of each panel the arrowheads indicate the position of molecular mass markers relevant to assessing the size of the expressed protein, and the correct band is denoted by a star for each polypeptide. Lane 1, Wyeth/IL-15; lane 2, Wyeth/IL-15/5Flu; and lane 3, Wyeth/mutIL-15/5Flu.



24-well clusters in triplicate. To provide APCs, syngeneic splenocytes from age-matched naive mice were infected with the H1N1 A/PR/8/34 virus at an MOI of 10 for 6 h and then irradiated (3000 rad) before being added at 1×10^6 cells per well. After 72 h of coculture, supernatants were harvested and the IFN- γ levels were measured using a commercial ELISA kit for mouse IFN γ (R&D Systems) according to the manufacturer's instructions. All samples were tested in triplicate in the assays.

Protection study

Animals immunized with the respective recombinant vaccinia viral vaccines were challenged intranasally with 100 LD₅₀ dose of HPAI H5N1 influenza virus 4 wk after the last vaccination. The dose of the influenza virus required for intranasal challenge experiments was predetermined. For challenge experiments, the H5N1 A/Ck/Indonesia/BL/2003 (clade 2.2) virus was used. The body weight of individual mice was measured daily for 14 days after administration of the challenge virus inoculum. All challenge experiments were conducted at animal biosafety level 3 in separate microisolators.

Histopathology and immunohistochemistry

Brain, lung, and spleen tissues harvested from representative animals from each experimental group challenged with HPAI H5N1 viruses on days 3 and 6 postchallenge were used for virus isolation and were titrated on MDCK cell monolayers. Some of the same tissue samples were also processed for histopathology and immunohistochemistry. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, cut into 4- μ m sections, and stained with H&E for histopathological evaluations. For immunohistochemistry, sequential slides were stained with a mixture of two mAbs directed against H5 hemagglutinin and NP influenza Ags. Antimouse Ab conjugated with HRP was then added for detection with diaminobenzidine as a substrate that forms red-brown precipitants. Nuclei of these sections were then counterstained with hematoxylin.

Statistical analysis

ANOVA was used to determine the effect of different immunization protocols on the magnitude of cellular and Ab responses as well as weight

changes after virus challenge. The Fisher's least significant difference test and pairwise *t* test (two samples, two-tailed Student *t* test) were used to compare pairs of immunization protocols, and significance levels were set at a value of $p = 0.05$.

Results

Validation of integrated H5N1 gene expression by vaccinia recombinants

In selecting the Ags to incorporate in our vaccinia-based vaccines, the objective was to develop a vaccine that is broadly efficacious against a spectrum of influenza A subtypes, if such a vaccine is achievable. In developing our MVA-based vaccine, H5 hemagglutinin and N1 neuraminidase genes from the A/Vietnam/1203/2004 strain were selected for integration and, thus, the MVA candidates are bivalent. In contrast, the Dryvax-based vaccines in the Wyeth strain of vaccinia were constructed to include, in addition to H5 hemagglutinin and N1 neuraminidase, the nucleoprotein NP and two matrix proteins M1 and M2, thus resulting in a pentavalent vaccine.

To define the contribution of IL-15 to vaccine-induced immunity and protection, we generated and tested in parallel an isogenic vaccine candidate with disabled IL-15 expression in both MVA-vectored bivalent vaccines (MVA/IL-15/HA/NA vs MVA/mutIL-15/HA/NA) and Wyeth-vectored pentavalent vaccines (Wyeth/IL-15/5Flu vs Wyeth/mutIL-15/5Flu) (where HA is hemagglutinin, NA is neuraminidase, mut is mutated, and Flu is influenza Ags).

To confirm the expression of integrated H5 hemagglutinin, N1 neuraminidase, nucleoprotein NP, and M1 matrix proteins by our Dryvax Wyeth strain-based pentavalent vaccine candidates, CV-1 monkey kidney cells were infected at a MOI of 10 and the infected cells were harvested 24 h post infection. Infected cell lysates were then subjected to SDS-PAGE and immunoblotted with specific Abs for each of the vaccine-encoded genes separately. As shown in Fig. 1, H5 hemagglutinin, N1 neuraminidase, M1 matrix protein and the NP Ags were abundantly expressed by our Wyeth/IL-15/5Flu and Wyeth/mutIL-15/5Flu vaccine candidates, and the levels of expression of these genes were similar between the two vaccine candidates. In the case of H5 hemagglutinin with the PSIQYR altered cleavage site sequence, the expressed protein still appeared to be processed in vaccinia-infected CV-1 cells to yield two major bands representing HA₁ (~55 kDa) and HA₂ (~27 kDa) with

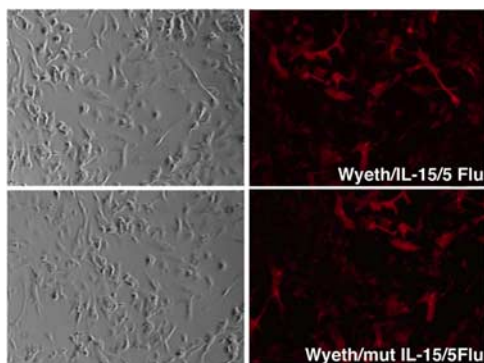


FIGURE 2. Detection of M2 protein expression by recombinant Wyeth vaccinia viruses by immunofluorescence microscopy. CV-1 cell monolayers infected with Wyeth/IL-15/5Flu or Wyeth/mutIL-15/5Flu reacted with an Ab specific for the M2 protein of the H5N1 influenza virus. Bound primary Ab was visualized using a rhodamine-conjugated secondary Ab. Left panels show the corresponding phase contrast images of the same fields.

Table I. Characterization of Wyeth/IL-15/5Flu vaccinia-expressed IL-15

Agent	Quantitation of IL-15 by ELISA	Bioactivity as Detected by Proliferative Index ^a
Wyeth/IL-15/5Flu	3,500 pg/ml	900.8
Wyeth/mutIL-15/5Flu	None detected	2.7
Culture medium	None detected	1.0

^a Proliferative index was calculated as the fold-increase in [³H]thymidine uptake above the medium control.

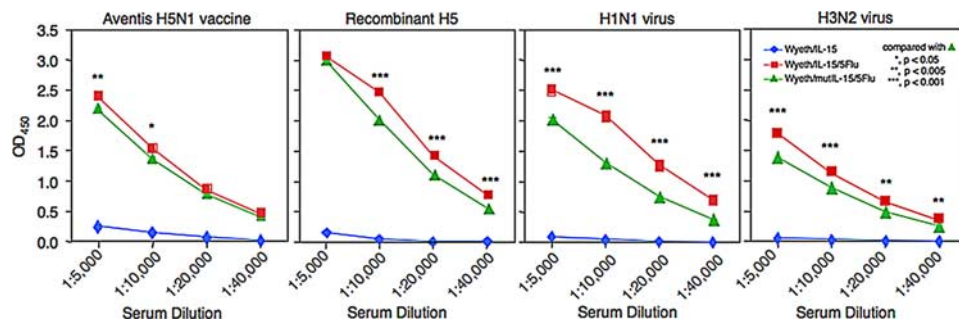


FIGURE 3. Influenza-specific Ab profiles of mice vaccinated with pentavalent influenza vaccines 4 wk after booster vaccination. Mice were vaccinated twice 4 wk apart s.c. with a dose of 1×10^7 PFU of virus. Sera were collected 4 wk later and pooled within each group. Wyeth/IL-15 virus-vaccinated animals served as a control. Influenza-specific Abs were detected by an ELISA, and the Ag preparation used as the plate-bound Ag in each ELISA is indicated on top of each panel.

some minor degradative products of the expressed hemagglutinin. Similarly, the N1 neuraminidase, which has a molecular mass of ~ 46 kDa, was expressed well, although a smaller band representing a polypeptide of ~ 40 kDa was also present in the infected lysates due to either degradation and/or premature termination during the synthesis of N1 neuraminidase. However, both the matrix protein M1 and the nucleoprotein NP, having molecular masses of ~ 30 and 60 kDa, respectively, were expressed without any apparent degradation of these proteins. Our bivalent MVA vaccine candidates, as was seen with the Wyeth vaccinia-based candidates, expressed both H5 hemagglutinin and N1 neuraminidase abundantly (data not shown). The expression of the M2 matrix protein was detected by immunofluorescence microscopy using a commercially available polyclonal Ab raised against a 13-aa peptide derived from the N terminus of the H5N1 M2 polypeptide (GenBank accession no. ABC74394). As shown in Fig. 2, the infected cells surrounding the viral plaques formed by both the Wyeth/IL-15/5Flu and the Wyeth/mutIL-15/5Flu recombinant vaccinia viruses displayed abundant expression of M2 diffusely distributed in the cytoplasm of infected cells. There is considerable interest in the tetrameric M2 proton-selective ion channel protein, not only as an attractive drug development target but also because of the highly conserved 24-aa extracellular domain (M2e) that can be potentially exploited to develop a broadly effective “universal vaccine” against influenza. However, many of the M2-specific Abs commercially available, including the 14C2 mAb, that have been raised against the H1N1 version of M2 failed to react with the M2 expressed by our vaccinia recombinants, which was derived from A/Ck/Indonesia/PA/2003 (GenBank accession no. AY651376). Similar failures of avian M2 recognition by nonavian M2-specific Abs have been reported previously (8). The expression of bioactive IL-15 was confirmed by the ability of infected culture supernatants to support the growth of an IL-15-dependent cell line (see Table I).

Immune profiles of mice vaccinated with recombinant vaccinia vaccines that express H5N1 influenza Ags

Groups of mice were vaccinated s.c. with each of the vaccinia recombinant vaccines. A second booster dose of vaccine was given to each group 4 wk later, and the serum Ab levels in the vaccinated animals were first determined by an ELISA 4 wk after the booster vaccination. A group of animals vaccinated with a recombinant Wyeth vaccinia virus that expresses IL-15 but not any of the H5N1 influenza Ags (Wyeth/IL-15) served as a control. For Ab titer determinations for each group, sera collected from all animals within a group were pooled. First we used the monovalent influenza subvirion vaccine (rgA/Vietnam/1203/2004) H5N1 manufactured by Aventis-Sanofi-Pasteur for human use as the capture Ag in the

ELISA. As shown in Fig. 3, both Wyeth/IL-15/5Flu group and Wyeth/mutIL-15/5Flu group sera displayed extremely high binding Ab titers for this Ag ($> 1/40,000$ dilution), whereas the control group did not display any meaningful reactivity at the dilutions tested. We also evaluated the binding Ab titers in vaccinated animals against the H5 hemagglutinin protein using recombinant H5 (A/Vietnam/1203/2004) as a capture Ag in the ELISA. The similarity of Ab titers generated against the H5 protein and the subvirion vaccine preparation suggests that the highest Ab titers elicited by our vaccine candidates are likely to be directed against the H5 hemagglutinin. However, when we used an H1N1 (A/PR/8/34)-infected MDCK cell lysate or a H3N2 (A/Aichi/2/68)-infected allantoic fluid as a source of multiple influenza Ags in the capture ELISA, the binding Ab levels to these non-H5 Ags (in the case of H1N1) or non-H5 and non-N1 (in the case of H3N2) were again quite high, suggesting that our vaccine candidates elicit broadly reactive humoral responses against multiple Ags in the vaccinated animals. It is noteworthy, as we have demonstrated previously with the other vaccine candidates we have developed (7, 9), that the incorporation of IL-15 resulted in an Ab response that was consistently higher, albeit modestly, when compared with the vaccine containing biologically inert mutant IL-15, and this modest difference in Ab levels was statistically significant. We also evaluated whether any M2-specific Abs were present in the pooled sera of Wyeth/IL-15/5Flu-vaccinated animals using an ELISA with a 19-aa peptide representing part of the extracellular domain of M2 polypeptide as the capture Ag. Unlike the H5 hemagglutinin Ag, M2 expressed by our vaccinia recombinant virus does not appear

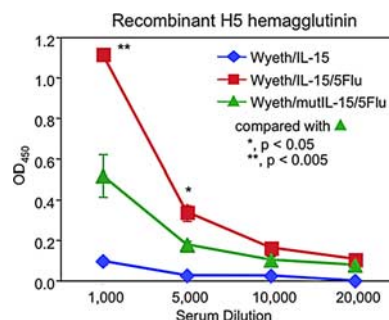


FIGURE 4. Influenza-specific Ab profiles of mice vaccinated with pentavalent vaccines 14 mo after booster vaccination are shown. Mice were vaccinated twice 4 wk apart s.c. with a dose of 1×10^7 PFU of virus. Animals were bled 14 mo later and sera were separated and pooled within each group. Wyeth/IL-15 virus-vaccinated animals served as a control. H5 hemagglutinin-specific Abs were detected by an ELISA with purified recombinant H5.

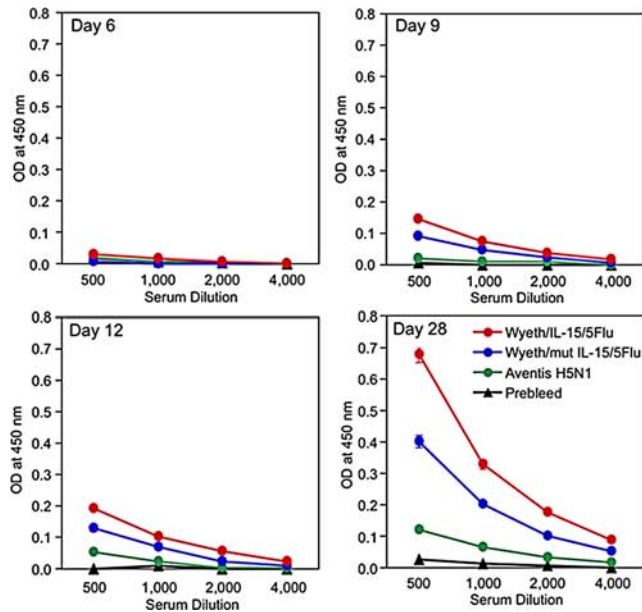


FIGURE 5. Induction kinetics of influenza-specific Ab responses in vaccinated mice. Mice were given a single s.c. dose (1×10^7 PFU of virus) of Wyeth/IL-15/5Flu or Wyeth/mutIL-15/5Flu. The Aventis vaccine (rgA/Vietnam/1203/2004) was given intramuscularly (100 μ l of vaccine per mouse injected to the gluteal region). Animals were bled every 3 days postvaccination and sera were separated and pooled within each group. H5 hemagglutinin-specific Abs were detected by an ELISA with recombinant H5 hemagglutinin as the plate-bound Ag. Data shown are selected time points where seroconversions occurred.

to be very immunogenic, because the M2-binding Ab titer was only 1,000 compared with >40,000 for the H5 hemagglutinin (data not shown). To assess the durability of our vaccine-induced Ab responses, these vaccinated mice were re-evaluated 14 mo after their second immunization. As shown in Fig. 4, even after 14 mo the influenza-specific Abs were still detectable in appreciable amounts in these vaccinated animals, although, as expected, some waning of this Ab response was apparent (~20-fold reduction over a period of 14 mo). Nonetheless, the sera from animals vaccinated with Wyeth/IL-15/5Flu still maintained significantly higher Ab levels than the mice vaccinated with Wyeth/mutIL-15/5Flu, supporting the notion that the presence of IL-15 during immune priming results in superior and long-lasting Ab responses.

Unlike seasonal influenza vaccines, a pandemic influenza vaccine is likely to be deployed in the face of an imminent influenza pandemic, and one of the crucial attributes of such a vaccine should be its ability to elicit a rapid protective immune response in unexposed vaccinees or a rapid immune response that can mitigate the clinical course of the disease if the individual is already exposed. Therefore, we compared the kinetics of influenza-specific Ab induction with our pentavalent vaccines and compared that with the Food and Drug Administration (FDA)-approved pandemic vaccine manufactured by Aventis-

Sanofi-Pasteur by administering a single dose of each vaccine to groups of mice and assessing the appearance of influenza-specific Abs in the vaccinated animals over a period of 4 wk, beginning on day 3 postvaccination. Although none of the groups had any detectable Abs on days 3 or 6 postvaccination, by day 9 postvaccination the animals vaccinated with Wyeth/IL-15/5Flu elicited detectable Abs (Fig. 5). However, in animals vaccinated with the FDA-approved Aventis-Sanofi-Pasteur vaccine, the appearance of detectable Abs occurred ~28 days postvaccination and even then the levels were significantly lower than those induced by our pentavalent vaccines, further confirming the superior immunogenicity of our vaccinia-based vaccines.

A potential concern with live vector-delivered vaccines has been whether the induction of neutralizing Ab responses against the vector could hamper multidose vaccination regimens of such vaccines. In Fig. 3, Ab levels were measured 4 wk after administering two doses of the vaccine, whereas in Fig. 5 the *bottom right panel* (labeled “Day 28”) shows the Ab levels 4 wk after a single dose of vaccination. A comparison of Ab levels in the two experiments clearly shows that effective multidose regimens are indeed possible with vaccinia-based vaccines such as our Wyeth/IL-15/5Flu to boost the immune responses induced by the primary vaccination. We have confirmed this finding in independent parallel experiments as well (data not shown).

Despite inducing high levels of binding Abs against H5N1 Ags, when we tested the same serum samples in a microneutralization assay against a panel of H5N1 virus isolates representing an array of clades/subclades, the neutralizing Ab titers in these samples were relatively low as depicted in Table II. The poor immunogenicity of H5 is well recognized, and the current guidelines mandate a hemagglutination inhibition titer of 40 or greater as acceptable for H5N1 vaccine candidates (10). It is interesting to note that the neutralizing Ab titer for both clade 1 viruses (A/Vietnam/1203/2004 and A/Vietnam/1194/2004) and a clade 2.2 virus (A/Ck/Indonesia/BL/2003) were identical, whereas no meaningful neutralizing Abs were detected against a clade 2.3 virus A/Vietnam/30850/2005. However, again as with the influenza Ag-binding Abs detected with ELISA in Fig. 3, the Wyeth/IL-15/5Flu vaccine elicited a slightly higher neutralizing Ab titer than the vaccine without IL-15 (Wyeth/mutIL-15/5Flu). It should be noted that the serum samples were tested under code, and the operator had no knowledge of sample identity. Our MVA-based bivalent vaccine candidates (MVA/IL-15/HA/NA and MVA/mutIL-15/HA/NA) elicited similar patterns of binding and neutralizing Abs in animals vaccinated with those candidate vaccines. For example, when we tested the sera from six mice vaccinated with MVA/mutIL-15/HA/NA individually in the microneutralization assay, four animals had a titer of 1/40 while two animals displayed a titer of 1/80 against A/Vietnam/1203/2004 virus, which compared well with the titer of 1/40 displayed by the pooled sera from Wyeth/mutIL-15/5Flu vaccinated mice. Collectively, these findings suggest that the IL-15-adjuvanted Wyeth/IL-15/5Flu vaccine confers a superior humoral

Table II. Serum-neutralizing Ab titers in vaccinated animals against different clades of H5N1

Vaccine	A/Vietnam/1203/2004 (clade 1)	A/Vietnam/1194/2004 (clade 1)	A/Ck/Indonesia/BL/2003 (clade 2.2)	A/Vietnam/30850/2005 (clade 2.3)
Wyeth/IL-15/5Flu	1:80	1:80	1:80	<1:10
Wyeth/mutIL-15/5Flu	1:40	1:40	1:40	<1:10
Wyeth/IL-5	<1:10	<1:10	<1:10	<1:10
Control BALB/c	<1:10	<1:10	<1:10	<1:10

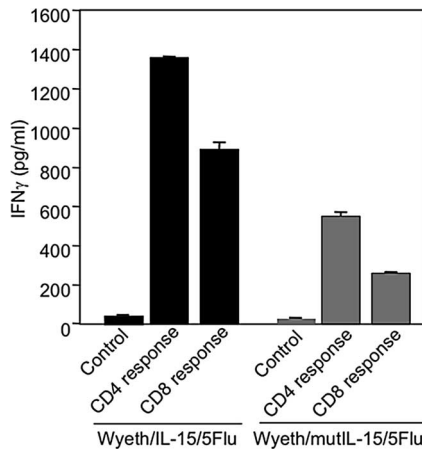


FIGURE 6. Cellular immune response against influenza Ags in vaccinated mice. Mice were s.c. vaccinated twice 4 wk apart with a dose of 1×10^7 PFU of virus. Three animals from each group were euthanized 8 days later and the splenocytes were harvested and pooled. From pooled splenocytes, CD4⁺ and CD8⁺ T lymphocytes were purified and then plated at 2×10^6 cells per well in triplicate. Age-matched, naive syngeneic splenocytes infected with the H1N1 (A/PR/8/34) virus was added to each well after irradiating (3000 rad) at 1×10^6 cells per well. Splenocytes (2×10^6 cells per well) from vaccinated animals cultured alone in parallel without irradiated H1N1-infected syngeneic splenocytes served as controls. After a 72-h period of coculture, IFN- γ levels in the culture supernatants were determined.

response against avian influenza H5N1 Ags that persists for a significant length of time.

The cellular immune responses against the influenza Ags in vaccinated animals were assessed by ex vivo culturing of CD4⁺ and CD8⁺ T cells purified from the spleens of vaccinated animals separately with syngeneic splenocytes infected with the A/PR/8/34 strain of H1N1 and by measuring the levels of secreted IFN- γ in the culture supernatants. As shown in Fig. 6, in the early phase of the immune response (8 days after the booster vaccination), although both CD4⁺ and CD8⁺ T cell responses were readily detectable in the vaccinated animals, there was a clear dominance in the CD4⁺ T cell response against influenza Ags regardless of whether the vaccine was adjuvanted with IL-15 or not. However, the Wyeth/IL-15/5Flu vaccine induced an ~ 2 -fold higher CD4⁺ and a 3-fold higher CD8⁺ T cell response in the vaccinated ani-

mals than the Wyeth/mut IL-15/5Flu. As expected, CD4⁺ and CD8⁺ T cells from mice vaccinated with Wyeth/IL-15, when cocultured with H1N1-infected syngeneic splenocytes, did not result in any increase in secreted IFN- γ above background. Similarly, when CD4⁺ and CD8⁺ T cells from Wyeth/IL-15/5Flu- or Wyeth/mutIL-15/5Flu-vaccinated mice were cocultured with uninfected irradiated syngeneic splenocytes, no increase in secreted IFN- γ was detected.

Vaccine induced protection against a lethal challenge of H5N1 virus

Having confirmed that our multivalent vaccinia-based vaccine candidates were capable of inducing neutralizing Abs against A/Ch/Indonesia/BL/2003, which is a clade 2.2 virus, to a level identical with that of A/Vietnam/1203/2004 (clade 1) from which the vaccine Ags were derived (Table II), we assessed the efficacy of our vaccinia-based influenza vaccines against a clade 2.2 H5N1 virus, A/Ck/Indonesia/BL/2003. As shown in Fig. 7, when a clade 2.2 H5N1 virus, A/Ck/Indonesia/BL/2003, was used to challenge the vaccinated mice 4 wk after the booster dose of vaccine, animals that were in the two control groups, namely the animals that were vaccinated with wild-type MVA or mock-vaccinated with PBS, displayed rapid loss of body weight starting 24 h postchallenge and succumbed to infection within a week after being challenged with the A/Ck/Indonesia/BL/2003 virus. In contrast, in animals vaccinated with any of the four vaccine candidates, namely, MVA/IL-15/HA/NA, MVA/mutIL-15/HA/NA, Wyeth/IL-15/5Flu, and Wyeth/mutIL-15/5Flu, no significant loss in body weight was observed during a period of 14 days after being inoculated with the A/Ck/Indonesia/BL/2003 virus. In fact, most animals continued to gain weight during this period of observation. Thus, all four vaccine candidates we have generated are 100% effective in preventing disease and death from a heterologous challenge with a different clade of H5N1 virus. When we examined the extent of viral replication in the mice challenged with A/Ck/Indonesia/BL/2003 (Table III), no culturable virus was detected in the lung, spleen, or brain tissues harvested from the vaccinated animals 6 days after intranasal administration of the A/Ck/Indonesia/BL/2003 virus, whereas in the MVA and PBS control groups the lung tissues yielded virus titers ranging from 10^4 to 10^7 PFU per 100 mg of tissue. In addition, in these control groups high viral titers were

FIGURE 7. Multivalent vaccinia virus-based H5N1 influenza vaccines protect mice against a heterologous lethal challenge with a HPAI virus. Mice were s.c. vaccinated twice 4 wk apart with a dose of 1×10^7 PFU of virus. Animals vaccinated with wild-type MVA or mock vaccinated with PBS in an identical manner served as controls. Vaccinated animals were challenged intranasally 4 wk after the second immunization with a 100 LD₅₀ dose of clade 2.2 H5N1 virus A/Ck/Indonesia/BL/2003. Body weights and survival were assessed daily for a period of 14 days. Individual animals within each group were designated 1–6 for identification.

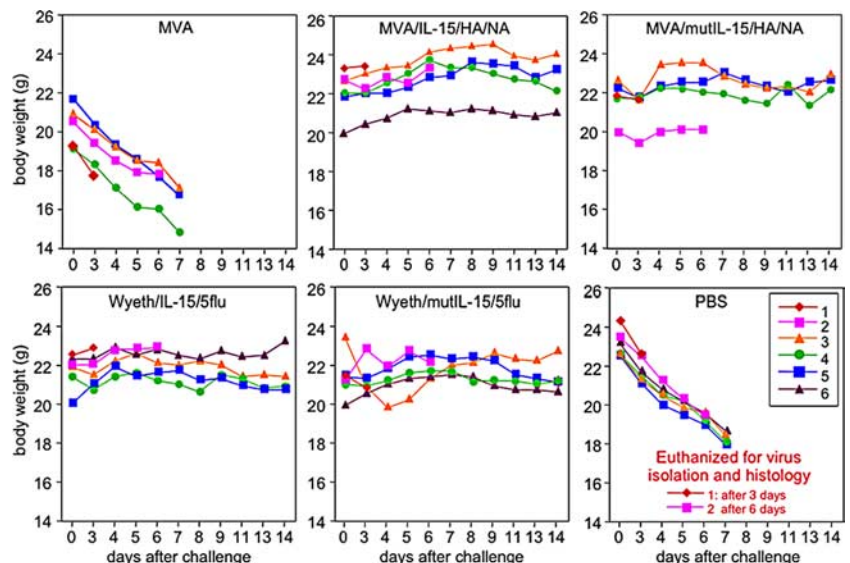


Table III. *Virus titers in tissues after challenge and survival of vaccinated mice*

Vaccine	TCID ₅₀ Virus Titer in Tissues ^a			Percentage (%) Survival ^b
	Lungs	Spleen	Brain	
MVA	6.06 ± 0.35	NCV	2.16 ± 1.98	0 (0/6)
MVA/IL-15/HA/NA	NCV	NCV	NCV	100 (6/6)
MVA/mutIL-15/HA/NA	NCV	NCV	0.3 ± 0.735	100 (6/6)
Wyeth/IL-15/5Flu	NCV	NCV	NCV	100 (6/6)
Wyeth/mutIL-15/5Flu	NCV	NCV	NCV	100 (6/6)
Control BALB/c	5.96 ± 1.02	NCV	2.10 ± 1.93	0 (0/6)

^a Virus titers were determined 3 days postchallenge and are expressed as the log₁₀ TCID₅₀/ml ± SD. NCV, No culturable virus present.

^b The number of survivors from the total number of animals per group is shown in parentheses.

detected in the brain tissues of many animals (see Table III). However, when we attempted to culture the virus three days after intranasal challenge, in addition to the two control groups the group of animals vaccinated with the bivalent MVA vaccine without the cytokine adjuvant IL-15 (MVA/mutIL-15/HA/NA) showed the presence of low levels of virus in the brain tissues but not in the lung or spleen tissues (see Table III). It should be noted, however, that no weight loss (Fig. 7) or any mortality (Table III) was seen in this group. Furthermore, when we looked for histopathological evidence of virus-induced lung injury in this group 3 days postchallenge (Fig. 8), minimal mononuclear cell infiltrations and thickening of alveolar septa were observed along with occasional perivascular cuffing as shown Fig. 8B. In marked contrast, in the animals that were vaccinated with the wild-type MVA virus there was widespread interstitial pneumonia with serous exudate-filled

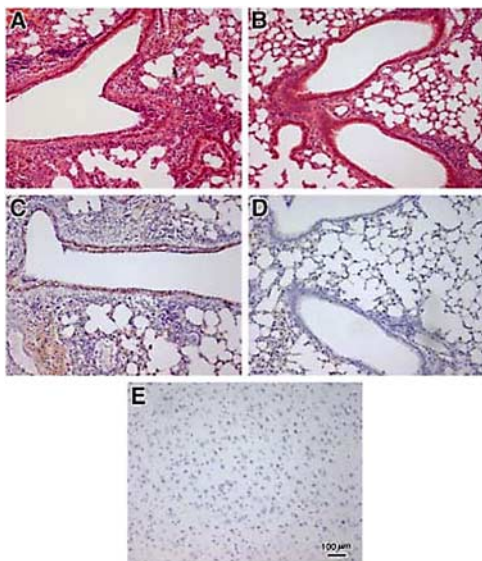


FIGURE 8. Histopathology and immunohistochemistry of select tissues of mice challenged with HPAI A/Indonesia/BLO3/2004 H5N1 virus. Mice were vaccinated and subsequently challenged as indicated in the legend to Fig. 7. Tissue sections were prepared from animals euthanized day 3 postchallenge from each group. *A*, Representative lung tissue from wild-type MVA vaccinated mice stained with H&E. *B*, Representative lung tissue from an animal vaccinated with MVA/mutIL-15/HA/NA; *C*, Lung tissue from an animal vaccinated with MVA and reacted with mAbs to NP and H5 viral Ags by immunohistochemistry. *D*, Lung tissue from an animal vaccinated with MVA/mutIL-15/HA/NA reacted with the same Abs used in *C*. *E*, Brain tissue section from an animal vaccinated with MVA/mutIL-15/HA/NA and reacted with mAbs to detect the presence of NP and H5 viral Ags by immunohistochemistry.

alveolar spaces in some areas, coalescing to form foci of inflammation and necrosis. The bronchiolar walls were thickened, as were alveolar septa with infiltrating macrophages and mononuclear cells, and the lung parenchyma showed areas of coagulation necrosis as well (Fig. 8A). We also examined lung and brain tissue sections for the presence of influenza viral Ags by immunohistochemistry using mAbs specific for nucleoprotein NP and H5 hemagglutinin. As shown in Fig. 8D, the lung tissues from animals vaccinated with MVA/mutIL-15/HA/NA failed to demonstrate any presence of viral Ags, whereas in the lung tissues from animals vaccinated with the wild-type MVA the extensive presence of influenza Ags was readily demonstrable by diffuse to granular red/brown staining in the epithelial cells lining the bronchiolar walls as well as the alveolar septa and pneumocytes (Fig. 8C). Intriguingly, despite yielding a cultivable virus, the brain tissues from animals vaccinated with MVA/mutIL-15/HA/NA failed to demonstrate any presence of NP or H5 Ags by immunohistochemistry (see Fig. 8E). Based on this negative evidence, one cannot be certain that vaccination with MVA/mutIL-15/HA/NA truly allows some limited viral replication initially in the brain, especially after administering a 100 LD₅₀ dose of A/Ck/Indonesia/BL/2003 virus or whether the one brain tissue sample that resulted in cultivable virus on day 3 postchallenge was in fact an aberrant event. We are currently pursuing studies to address this possibility. An equally important observation was that mice vaccinated with MVA/IL-15/HA/NA, Wyeth/IL-15/5Flu, or Wyeth/mutIL-15/5Flu not only failed to yield any cultivable virus following challenge but also demonstrated no histopathological lesions in the tissues examined or showed immunohistochemical evidence for the presence of influenza Ags (data not shown).

Discussion

Our vaccine development strategy involved three elements, namely the selection of a live delivery vector, the incorporation of a repertoire of antigenic targets to achieve broad cross protection, and the incorporation of a molecular adjuvant to enhance the breadth and durability of vaccine-induced immune responses. In designing our multivalent H5N1 influenza vaccine candidates, we opted to use a live viral vector vaccinia virus for a number of reasons as outlined below. Vaccinia virus has a proven record of efficacy and safety and carries the feasibility of large-scale manufacture in a relatively short time frame. Vaccinia recombinants are genetically stable and possess the intrinsic capacity to induce multiple arms of the immune system conferring robust and sustainable immune responses (11). Importantly, unlike in the case of Ad5-vectored vaccines, pre-existing Abs to vaccinia virus in the general population that could impede vaccine efficacy are likely to be less of a problem, because smallpox vaccinations ended in the early 1970s globally (12). In addition, vaccinia recombinants expressing individual influenza genes from various subtypes such as the hemagglutinin gene, nuclear protein gene, or matrix M1 gene have been shown to protect animals from a subsequent lethal challenge of virulent influenza viruses (13, 14). We selected the replication-competent, FDA-licensed smallpox Dryvax vaccine (Wyeth strain) as the backbone for integrating H5N1 avian influenza viral genes, because >1 billion people have been vaccinated with the Dryvax vaccine during the smallpox eradication campaigns four to five decades ago and recently the FDA has approved its production in Vero cell substrates, thus enabling a rapid scale-up of Dryvax-based vaccine manufacturing (see U.S. Food and Drug Administration news report for September 1, 2007; www.fda.gov/bbs/topics/NEWS/2007/NEW01693.html). We have also made a set of H5N1 influenza vaccine candidates in the replication-deficient MVA backbone. MVA is under consideration for

licensure as an alternate to the Dryvax smallpox vaccine, and MVA-based recombinant vaccine candidates against a number of infectious diseases such as HIV, malaria, and tuberculosis as well as certain malignancies such as prostate cancer are currently in human trials (reviewed in Ref. 11). These recombinant MVA vaccine candidates are manufactured in a primary chick embryo fibroblast cell substrate, which is the only cell substrate for MVA vaccine production approved by regulatory agencies such as the FDA. In our view, although MVA is better suited for contemporary populations having large numbers of immunodeficient individuals due to HIV infections or organ transplants and individuals with atopic skin diseases for whom the administration of Dryvax vaccine is contraindicated, an MVA-based vaccine against HPAI strains such as the H5N1 strains is likely to face the same large-scale production constraints associated with the traditional egg-based influenza vaccine production during a pandemic. The fact that MVA vaccine production depends on a continuous supply of embryonated eggs for primary chick embryo fibroblasts limits the utility of such a vaccine against a HPAI that is also likely to decimate poultry flocks during an influenza pandemic in people. But if the MVA-based vaccine candidate is of broad cross reactivity, then pre-pandemic stockpiling of such a vaccine could overcome those production constraints.

The hemagglutinin protein is the principal target of protective Abs. The neuraminidase protein is the second most abundant surface protein and displays less antigenic variation in comparison with hemagglutinin, and the Abs generated against neuraminidase have been shown to be protective and broadly cross-reactive (15). To achieve broad cross-reactivity in a vaccine, the internal and structural proteins constitute attractive targets because of their relatively invariant nature across all subtypes of influenza A viruses and also because they are the principal targets of CTL activity in humans (16, 17); hence our rationale to incorporate these NP, M1, and M2 genes in our pentavalent vaccine.

The selection of the IL-15 cytokine as an immune-enhancing adjuvant was based on its critical role in orchestrating both innate and adaptive immune responses. IL-15 is pivotally involved in the maintenance of CD8⁺ memory cells and NK cells without any inductive effects on CD4⁺/CD25⁺ T regulatory cells (reviewed in Ref. 18). In addition, IL-15 inhibits activation-induced cell death of T lymphocytes that could potentially contribute to a more intense and prolonged immune response. We have previously demonstrated that immune responses elicited in the presence of IL-15 give rise to long-lived, Ag-specific CD8⁺ memory T cells that display enhanced avidity to their cognate Ags (7, 19). It has been documented that CD8⁺ T cells that display higher avidity to their cognate viral or tumor Ags clear viral infections or tumors more efficiently (20, 21). Furthermore, IL-15 also enhances Ab responses to vaccine Ags when coexpressed with such vaccine Ags (7, 9, 22). All of these attributes of IL-15 favored our selection of this cytokine as an immune-enhancing molecular adjuvant in our vaccines against H5N1 Ags that are known to be poorly immunogenic. Equally important is the observation that the Dryvax vaccine, which can cause severe vaccinal disease in immunodeficient hosts, loses its virulence markedly with the incorporation of IL-15 as we have shown previously (5). Thus, our selection of IL-15 to be integrated into our Dryvax Wyeth strain-based pentavalent H5N1 vaccine facilitated achieving both immune enhancement as well as attenuation of the virulence of these vaccines.

Similar to our observations made here, it should be noted that the live attenuated influenza A H5N1 vaccine of Suguitan et al. (23), as well as almost all live vector-delivered H5N1 vaccines reported to date, have demonstrated some level of heterologous protection even in the absence of detectable neutralizing Abs (re-

viewed in Ref. 3), attesting to the superiority of live vaccines and the potency and breadth of the protective cellular immune responses such vaccines can induce (24–26). In contrast, the currently licensed subvirion H5N1 vaccine (rgA/Vietnam/1203/2004) for pandemic use is incapable of inducing any meaningful cross-clade neutralizing Abs even with the recommended regimen of two doses of the vaccine unless administered with a potent adjuvant such as MF59 (27). Equally worrisome is the observation, as we have shown with the licensed H5N1 subvirion vaccine in Fig. 5, that the induction kinetics of Ab responses take almost 4 wk to achieve a level that is barely measurable after a single dose, and this does not inspire much confidence as to its efficacy in a pandemic situation. In designing effective pandemic influenza vaccines our goal should be to develop a vaccine that can prevent death and serious disease with a single dose because, with an impending pandemic, multidose vaccination regimens are not likely to be realistic. Therefore, in our ongoing studies we are evaluating the efficacy of our vaccinia-based vaccines with a single dose regimen in parallel with the licensed H5N1 vaccine. Furthermore, our Wyeth strain-based pentavalent vaccine expresses three highly conserved Ags, namely the NP, M1, and M2 proteins, thus warranting evaluation of its potential as a “universal influenza vaccine” in our ongoing studies as well.

The true impact of integrated IL-15 on the residual virulence of the Wyeth strain in humans can be assessed only from a carefully planned large-scale trial. Although the fact that vaccination with both Wyeth/IL-15/5Flu and Wyeth/mutIL-15/5Flu conferred sterile protection against clade 2.2 H5N1 challenge precluded a definitive critical role for IL-15 adjuvantation, it should be noted that the incorporation of IL-15 in our vaccinia-based vaccines resulted in the improvement of both humoral and cellular responses against H5N1 Ags quantitatively as shown in Figs. 3–6. In our ongoing studies we are continuing to assess the impact of IL-15-mediated enhanced immunogenicity on protection by using higher doses of homologous and heterologous H5N1 challenge viruses. The bivalent MVA-based HA/NA vaccine, which, as we have shown in this study, appears to be equally robust, could be used for individuals for whom the Wyeth strain-based pentavalent vaccine is contraindicated in a manner analogous to that in which MVA was used as a prevaccine in individuals for whom the Dryvax vaccine was contraindicated during the last stages of smallpox vaccination campaigns.

In summary, we have developed two sets of multivalent vaccine candidates against H5N1 using a vaccinia live vector delivery platform with a proven track record in human use that confer sterile protection against multiple clades of HPAI H5N1 that can be manufactured with existing production capabilities, thus ensuring their rapid deployability in the event of an influenza pandemic to protect humanity.

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Disclosures

The authors have no financial conflict of interest.

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