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# Vaccinia virus-based multivalent H5N1 avian influenza vaccines adjuvanted with IL-15 confer sterile cross-clade protection in mice[1]

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## Abstract

The potential for a global influenza pandemic remains significant with epidemiologic and ecologic indicators revealing the entrenchment of 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 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 antibodies and robust cellular immune responses in vaccinated mice and conferred sterile cross-clade protection when challenged with H5N1 virus of a different clade. In addition to having the potential as a universal influenza vaccine, in the event of an impending pandemic, the Wyeth/IL-15/5Flu is also readily amenable for bulk production to cover the global population. For those individuals for whom the use of 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.

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**Keywords**

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## Introduction

The past century was witness to three influenza pandemics that ravaged humanity. The "Spanish Flu" of 1918 caused by the emergence of influenza A H1N1 virus, killed according to the most modest estimates over 30 million people world-wide, followed by "Asian Flu" in 1958 with the appearance of H2N2 that led to another one million deaths (1). The last pandemic of the 20$^{th}$ century was in 1968 caused by influenza A H3N2 dubbed "Hong Kong Flu" that reportedly killed 800,000 people in just 6 weeks (1). The appearance of the highly pathogenic avian influenza A (HPAI) H5N1 virus in Hong Kong in 1997 was thought to be a harbinger of the first influenza pandemic of the 21$^{st}$ 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 over 60% (3).

With the looming threat of a global influenza pandemic, there is general consensus that the most effective public health intervention in curbing the morbidity, mortality and the economic catastrophe associated with such a pandemic is an effective vaccine. Such a vaccine should be efficacious, safe and be amenable to 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 that is manufactured by Aventis-Sanofi-Pasteur (Swiftwater, PA) (4). The vaccine is an inactivated, detergent extracted subvirion formulation, and has been added to the US 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 and a number of potential candidate vaccines have been developed and some of these candidates are being evaluated in clinical trials (reviewed in ref 5). We have taken the view that a multi-valent live vector-delivered vaccine carrying multiple immunogenic influenza polypeptides that have 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 5 genes of H5N1 influenza A virus, namely, the H5 hemagglutinin gene, N1 neuraminidase gene, nucleoprotein NP gene, and genes encoding the two matrix polypeptides M1 and M2 along with the cytokine IL-15 on the backbone of Wyeth strain of vaccinia or only the two surface genes H5 hemagglutinin, and N1 neuraminidase with IL-15 on the 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 DC. It also plays a role in B cell differentiation, maturation and antibody secretion thus making IL-15 an ideal candidate for incorporation as a molecular adjuvant in vectored vaccines. Our vaccine candidates in pre-clinical 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 a broadly effective pandemic influenza vaccine.

## 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 (6). From the extracted viral RNA, cDNA was synthesized by standard RT-PCR and the coding segment of the hemagglutinin, neuraminidase and nucleoprotein were amplified individually by polymerase chain reaction (PCR). The 5' primers contained a synthetic early-late vaccinia promoter added prior to the initiator codon ATG and the 3' primer contained a vaccinia transcription terminator sequence TTTTTCT added after the gene specific translation terminator codon for each of the genes amplified. In the case of the hemagglutinin gene, the $H_0$ 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 TTTTTCT vaccinia transcription terminator added 3' to the natural translation stop codon of the respective gene. The coding region DNA sequences were derived from the GenBank entry AY651376 for M1 and M2 (A/CK/Indonesia/PA/2003, H5N1). The coding segment of human IL-15 gene with 5' vaccinia early-late promoter and a 3' TTTTTCT transcriptional terminator sequence has been described previously (7). 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 (Marietta, PA). Modified vaccinia virus Ankara (an isolate made in 1974 before the bovine spongiform encephalopathy era) was kindly provided by Dr. Bernard Moss from the National Institute of Allergy and Infectious Diseases. To create recombinant vaccinia viruses pTFHA transfer vector with a 1.8 Kb DNA fragment encompassing the hemagglutinin gene of vaccinia and *E. coli gpt* gene was used as described earlier (7). Wyeth recombinant viruses with 5 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 was used to create MVA recombinant viruses. Recombinant viruses were generated by standard procedures as described previously (7) 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 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% CPE, infected monolayers were harvested and cell pellets were resuspended in RIPA 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 PVDF membranes for immunoblotting. The following primary antibodies were used for the detection of influenza antigens: polyclonal rabbit antibody for N1 neuraminidase (cat# 21304-100); polyclonal rabbit antibody for hemagglutinin (cat# 21297-100); polyclonal rabbit antibody for nuclear protein NP (cat#21008-100) and polyclonal goat antibody for M1 (cat# 21008-100). The above antibodies were purchased from Abcam Inc, Cambridge, MA and were used at a final concentration of 1 mcg/ml.

### Immunofluorescence

For detection of M2 expression, CV-1 cells were grown in LAB-TEK borosilicate chamber slides and infected with Wyeth recombinants at an MOI of 0.1 for 24 hours. Infected cells were fixed in ethanol/acetone and then reacted with a rabbit polyclonal antibody specific for the M2 protein of H5N1 virus (cat# 4333) from ProSci Inc., Poway, CA. The detection antibody was a rhodamine conjugated anti-rabbit antibody.

### Detection of IL-15 activity

CV-1 cells were infected with vaccinia virus at a multiplicity of infection of 10 and infected cells were cultured for 3 days prior to 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, Minneapolis, MN). Bioactivity was determined by the ability of supernatants to support the growth of IL-2/IL-15-dependent NK-92 cell line as reported previously (8). After addition of test supernatants cells were incubated for 48 hours then pulsed with 1 microCi/ml of $^{3}$H-thymidine for 6 additional hours. Triplicate samples were counted by scintillation. Cells incubated with media alone served as a negative control. The proliferative index was calculated as the fold-increase in $^{3}$H-thymidine uptake above the media control.

### Mice and Immunizations

Specific pathogen free Female BALB/c mice (6–10 weeks old) were used for immunization studies. All animal procedures were carried out under institutionally approved protocols. Mice were immunized subcutaneously at the base of the tail with $1\times10^{7}$ plaque-forming units (pfu) of vaccinia virus in a volume of 100 microliters. A second booster dose was given 4 weeks later. Each experimental group consisted of 6 animals. In certain experiments, for comparative analysis mice were given a single dose of FDA-approved Aventis vaccine (rgA/Vietnam/1203/2004) intramuscularly (100 microliters containing 3 mcg of vaccine per mouse injected to the gluteal region).

### Assays for immune responses

An enzyme linked immunosorbent assay (ELISA) was used to measure the levels of influenza antigen-binding antibodies in sera collected from vaccinated mice, essentially as described previously except for the antigen preparations used for coating ELISA plates (9). For coating ELISA plates, three different preparations of influenza antigens were used depending on the experiment. For certain experiments, purified baculovirus-expressed recombinant H5 hemagglutinin was used at a concentration of 1 mcg/ml from Protean Sciences Corp., Meridian CT (cat#3006). We also used H1N1-infected MDCK cell culture supernatants having a $1\times10^{6}$ pfu/ml virus titer after diluting 7-fold in carbonate coating buffer. H3N2 A/Aichi/2/68 infected allantoic fluid was also used. In some experiments we used monovalent subvirion vaccine (rgA/Vietnam/1203/2004) manufactured by Aventis Pasteur Inc., with a 30 mcg/ml standardized hemagglutinin content after diluting 20-fold in carbonate coating buffer. Neutralizing antibody titers were determined by a microneutralization assay using MDCK cell monolayers with a 100 $TCID_{50}$ infectivity 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 antigens, an *in vitro* co-culture 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 Biotech, Auburn, CA according to manufacturer's instructions. Purified cells were plated at $2\times10^{6}$ cells per well in 24 well clusters in triplicate. To provide antigen presenting cells syngeneic splenocytes from age matched naïve mice were infected with H1N1 A/PR/8/34 virus at an MOI of 10 for six hours and then irradiated (3000 rad) prior to adding

at $1\times10^6$ cells per well. After 72 hours of co-culture, supernatants were harvested and the interferon gamma (IFN γ) levels were measured using a commercial ELISA kit for mouse IFN γ (R&D Systems, Minneapolis, MN) 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 $LD_{50}$ dose of HPAI H5N1 influenza virus 4 weeks after the last vaccination. The dose of the influenza virus required for intranasal challenge experiments was predetermined. For challenge experiments 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 carried out in ABSL 3 biocontainment 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 day 3 and day 6-post challenge 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 and sectioned at 4 micrometers and stained with hematoxylin-eosin for histopathological evaluations. For immunohistochemistry, sequential slides were stained with a mixture of two monoclonal antibodies directed against H5 hemagglutinin and NP influenza antigens. Anti-mouse antibody conjugated with horseradish peroxidase was then added for detection with diamino-benzidine as a substrate that forms red-brown precipitants. Nuclei of these sections were then counter-stained with hematoxylin.

### Statistical analysis

Analysis of variance was used to determine the effect of different immunization protocols on the magnitude of cellular and antibody responses as well as weight changes after virus challenge. The Fisher's least-significance-difference test and pair wise t-test (two samples, two-tailed Student t test) were used to compare pairs of immunization protocols and significance levels were set at a $P$ value of 0.05.

## Results

### Validation of integrated H5N1 gene expression by vaccinia recombinants

In selecting the antigens 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 were selected for integration and thus MVA candidates are bivalent. In contrast, the Dryvax based vaccines in 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 versus MVA/mutIL-15/HA/NA) and Wyeth vectored pentavalent vaccines (Wyeth/IL-15/5Flu versus Wyeth/mutIL-15/5Flu).

To confirm the expression of integrated H5 hemagglutinin, N1 neuraminidase, nucleoprotein NP, and M1 matrix proteins by our Dryvax Wyeth strain based petavalent vaccine candidates, CV-1 monkey kidney cells were infected at a multiplicity of infection (MOI) of 10 and the infected cells were harvested 24 hours post infection. Infected cell lysates were then subjected to SDS-PAGE and immunoblotted with specific antibodies for each of the vaccine encoded genes separately. As shown in Fig. 1, H5 hemagglutinin, N1 neuraminidase, M1 matrix protein and the NP antigens 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 vacciniainfected CV-1 cells to yield two major bands representing $HA_1$ (~55 kDa) and $HA_2$ (~27 kDa) with some minor degradative products of the expressed hemagglutinin. Similarly, the N1 neuraminidase which has a molecular weight of approximately 46 kDa was expressed well although a smaller band representing a polypeptide of approximately 40 kDa was also present in the infected lysates either due to degradation and/or premature termination during the synthesis of N1 neuraminidase. However, both matrix protein M1 and nucleoprotein NP having molecular weights of approximately 30 kDa and 60 kDa respectively, were expressed without any apparent degradation of these proteins. Our bivalent MVA vaccine candidates as was seen with Wyeth vaccinia based candidates expressed both H5 hemagglutinin and N1 neuraminidase abundantly (data not shown). The expression of M2 matrix protein was detected by immunofluorescence microscopy using a commercially available polyclonal antibody raised against a 13 amino-acid peptide derived from the N-terminus of H5N1 M2 polypeptide (GenBank accession number ABC74394). As shown in Fig. 2, the infected cells surrounding viral plaques formed by both Wyeth/IL-15/5Flu and 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 amino acid extracellular domain (M2e) that can be potentially exploited to develop a broadly effective “universal vaccine” against influenza. However, many of the M2-specific antibodies commercially available including the 14C2 MAb that have been raised against the H1N1 version of M2 failed to react with M2 expressed by our vaccinia recombinants which was derived from A/Ck/Indonesia/PA/2003 (GenBank accession number AY651376). Similar failures of avian M2 recognition by non-avian M2 specific antibodies have been reported previously (10). 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 antigens

Groups of mice were vaccinated subcutaneously with each of the vaccinia recombinant vaccines. A second booster dose of vaccine was given to each group 4 weeks later and the serum antibody levels in the vaccinated animals were first determined by an ELISA assay 4 weeks after the booster vaccination. A group of animals vaccinated with a recombinant Wyeth vaccinia virus that expresses IL-15 but not any H5N1 influenza antigens (Wyeth/IL-15) served as a control. For antibody 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 Inc., for human use as the capture antigen in the ELISA assay. As shown in Fig 3, both Wyeth/IL-15/5Flu group and Wyeth/mutIL-15/5Flu group sera displayed extremely high binding antibody titers for this antigen (over 1;40,000 dilution) whereas the control group did not display any meaningful reactivity at the dilutions tested. We also evaluated the binding antibody titers in vaccinated animals against the H5 hemagglutinin protein using recombinant H5 (A/Vietnam/1203/2004)

as a capture antigen in the ELISA assay. The similarity of antibody titers generated against the H5 protein and the subvirion vaccine preparation suggests that the highest antibody 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 antigens in the capture ELISA, the binding antibody levels to these non-H5 antigens (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 antigens in the vaccinated animals. It is noteworthy as we have demonstrated previously with other vaccine candidates we have developed (9,11), the incorporation of IL-15 resulted in an antibody response that was consistently higher albeit modestly when compared to the vaccine containing biologically inert mutant IL-15 and this modest difference in antibody levels was statistically significant. We also evaluated whether any M2 specific antibodies were present in the pooled sera of Wyeth/IL-15/5Flu vaccinated animals using an ELISA with a 19 amino acid peptide representing part of the extracellular domain of M2 polypeptide as the capture antigen. Unlike the H5 hemagglutinin antigen, M2 expressed by our vaccinia recombinant virus does not appear to be very immunogenic because the M2-binding antibody titer was only 1000 compared to over 40,000 for the H5 hemaglutinin (data not shown). To assess the durability of our vaccine induced antibody responses, these vaccinated mice were re-evaluated 14 months after their second immunization. As shown in Fig. 4, even after 14 months, the influenza-specific antibodies were still detectable in appreciable amounts in these vaccinated animals although as expected some waning of this antibody response was apparent (approximately about 20-fold reduction over a period of 14 months). Nonetheless, the sera from animals vaccinated with Wyeth/IL-15/5Flu still maintained significantly higher antibody 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 antibody 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 antibody induction with our pentavalent vaccines and compared that with the 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 antibodies in the vaccinated animals over a period of 4 weeks beginning day 3 post vaccination. Although none of the groups had any detectable antibodies on day 3 or on day 6 post vaccination, by day 9 post vaccination, animals vaccinated with Wyeth/IL-15/5Flu elicited detectable antibodies (Fig. 5). However, in animals vaccinated with the FDA-approved Aventis-Sanofi-Pasteur vaccine, the appearance of detectable antibodies occurred around 28 days post vaccination 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 antibody responses against the vector could hamper multi-dose vaccination regimens of such vaccines. In Fig. 3, antibody levels were measured 4 weeks after administering two doses of the vaccine whereas in Fig. 5, the last panel shows the antibody levels 4 weeks after a single dose of vaccination. A comparison of antibody levels in the two experiments clearly shows that effective multi-dose 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 antibodies against H5N1 antigens, when we tested the same serum samples in a micro-neutralization assay against a panel of H5N1 virus isolates representing an array of clades/subclades, the neutralizing antibody 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 (HI) titer of 40 or greater as acceptable for H5N1 vaccine candidates (12). It is interesting to note that the neutralizing antibody 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 antibodies were detected against a clade 2.3 virus A/Vietnam/30850/2005. However, again as with influenza antigen-binding antibodies detected with ELISA in Fig. 3, the Wyeth/IL-15/5Flu vaccine elicited a slightly higher neutralizing antibody 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 bi-valent vaccine candidates (MVA/IL-15/HA/NA and MVA/mutIL-15/HA/NA) elicited similar patterns of binding and neutralizing antibodies 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 response against avian influenza H5N1 antigens that persists for a significant length of time.

The cellular immune responses against the influenza antigens 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 measuring the levels of secreted interferon gamma (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 antigens regardless of whether the vaccine was adjuvanted with IL-15 or not. However, Wyeth/IL-15/5Flu vaccine induced an approximately 2-fold higher $CD4^+$ and a 3-fold higher $CD8^+$ T cell response in the vaccinated animals than the Wyeth/mut IL-15/5Flu. As expected $CD4^+$ and $CD8^+$ T cells from mice vaccinated with Wyeth/IL-15 when co-cultured with H1N1-infected syngeneic splenocytes did not result in any increase in secreted IFN-γ above background. Similarly, $CD4^+$ and $CD8^+$ T cells from Wyeth/IL-15/5Flu or Wyeth/mutIL-15/5Flu vaccinated mice when co-cultured 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 antibodies against A/Ch/Indonesia/BL/2003 which is a clade 2.2 virus to a level identical to that of A/Vietnam/1203/2004 (clade 1) from which the vaccine antigens 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 weeks 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 hours post-challenge and succumbed to infection within a week after being challenged with 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 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 A/Ck/Indonesia/BL/2003 virus whereas in the MVA and PBS control groups, 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 detected in the brain tissues of many animals (see Table III). However, when we attempted to culture 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, 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 three days post-challenge (Fig. 8), minimal mononuclear cell infiltrations and thickening of alveolar septa were observed along with occasional perivascular cuffing as shown Fig. 8, panel B. In marked contrast, the animals that were vaccinated with the wild-type MVA virus, there was widespread interstitial pneumonia with serous exudate-filled 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. 8, panel A). We also examined lung and brain tissue sections for the presence of influenza viral antigens by immunohistochemistry using monoclonal antibodies specific for nucleoprotein NP and H5 hemagglutinin. As shown in panel D of Fig. 8, the lung tissues from animals vaccinated with MVA/mutIL-15/HA/NA failed to demonstrate any presence of viral antigens, whereas in the lung tissues from animals vaccinated with the wild-type MVA, extensive presence of influenza antigens was readily demonstrable as diffuse to granular red/brown staining, including in the epithelial cells lining the bronchiolar walls as well as alveolar septa and pneumocytes (Fig. 8, panel C). Intriguingly, despite yielding cultivable virus, the brain tissues from animals vaccinated with MVA/mutIL-15/HA/NA failed to demonstrate any presence of NP or H5 antigens by immunohistochemistry (see Fig. 8, panel E). 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 $LD_{50}$ dose of A/Ck/Indonesia/BL/2003 virus or whether the one brain tissue sample that resulted in cultivable virus on day 3 post challenge 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 antigens (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 multi-valent 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 sustainable immune responses (13). Importantly, unlike in the case of Ad5

vectored vaccines, pre-existing antibodies in the general population to vaccinia virus that could impede vaccine efficacy is likely to be less of a problem since smallpox vaccinations ended in the early 70's globally (14). 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 (15,16). We selected the replication competent FDA licensed smallpox Dryvax vaccine (Wyeth strain) as the backbone for integrating H5N1 avian influenza viral genes because over a billion people have been vaccinated with the Dryvax vaccine during the smallpox eradication campaigns 4–5 decades ago, and recently the FDA has approved its production in Vero cell substrates thus enabling rapid scale up of manufacturing Dryvax based vaccines (17). 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, tuberculosis as well as certain malignancies such as prostate cancer are in human trials currently (reviewed in ref 13). These recombinant MVA vaccine candidates are manufactured in primary chick embryo fibroblast cell substrate which is the only approved cell substrate for MVA vaccine production 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 a highly pathogenic avian influenza (HPAI) strains such as 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 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 antibodies. The neuraminidase protein is the second most abundant surface protein and displays less antigenic variation in comparison to hemagglutinin and the antibodies generated against neuraminidase have been shown to be protective and broadly cross-reactive (18). In order 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 being the principal targets of CTL activity in humans (19,20), hence our rationale to incorporate these NP, M1 and M2 genes in our pentavalent vaccine.

The selection of 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 21). 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 antigen specific CD8+ memory T cells that display enhanced avidity to their cognate antigens (9,22). It has been documented that $CD8^+$ T cells that display higher avidity to their cognate viral or tumor antigens clear viral infections or tumors more efficiently (23,24). Furthermore, IL-15 also enhances antibody responses to vaccine antigens when co-expressed with such vaccine antigens (9,11,25). All of these attributes of IL-15 favored our selection of this cytokine as an immune-enhancing molecular adjuvant in our vaccines against H5N1 antigens that are known to be poorly immunogenic. Equally important is the observation that the Dryvax vaccine that can cause severe vaccinal disease in immunodeficient hosts, loses its virulence markedly with the incorporation of IL-15 as we have shown previously (7). 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 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. (26), 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 antibodies (reviewed in ref 5) attesting to the superiority of live vaccines and the potency and the breadth of protective cellular immune responses such vaccines can induce (27–29). In contrast, the currently licensed subvirion H5N1 vaccine (rgA/Vietnam/1203/2004) for pandemic use is incapable of inducing any cross-clade neutralizing antibodies even with the recommended regimen of two doses of the vaccine unless administered with a potent adjuvant such as MF59 (30). Equally worrisome is the observation as we have shown with the licensed H5N1 subvirion vaccine in Fig. 5, the induction kinetics of antibody responses take almost 4 weeks 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 multi-dose 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 antigens, namely NP, M1 and M2 proteins abundantly 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 Wyeth strain in humans can be assessed only from a carefully planned large-scale trial. The fact that vaccination with Wyeth/IL-15/5Flu and Wyeth/mutIL-15/5Flu both conferred sterile protection against clade 2.2 H5N1 challenge, although 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 antigens quantitatively as shown in Figure 3 through Figure 6. In our ongoing studies we are continuing to assess the impact of IL-15 mediated enhanced immunogenicity on protection using higher doses of homologous and heterologous H5N1 challenge viruses. The bivalent MVA-based HA/NA vaccine which appears to be equally robust as we have shown here could be used for individuals for whom the Wyeth strain based pentavalent vaccine is contraindicated in a manner analogous to when 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 which can be manufactured with existing production capabilities ensuring their rapid deployability in the event of an influenza pandemic to protect the humanity.

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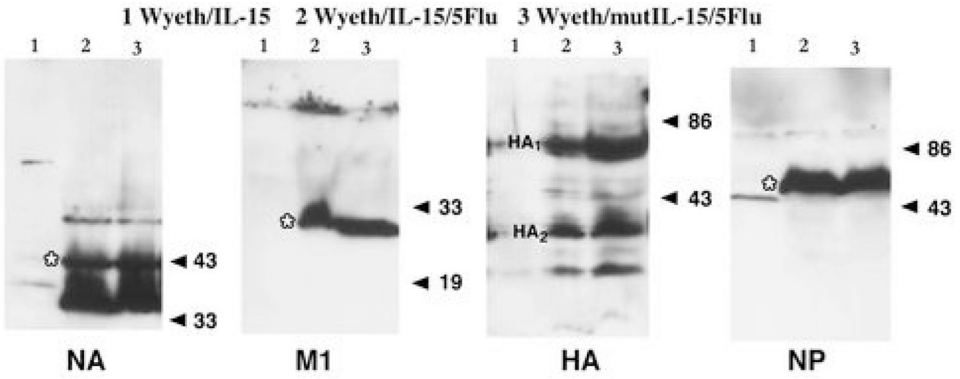


**Figure 1.**

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 antibodies specific for NA, M1, HA and NP proteins of influenza A virus. Listed on the right side of each panel, arrowheads indicate the position of molecular weight 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.

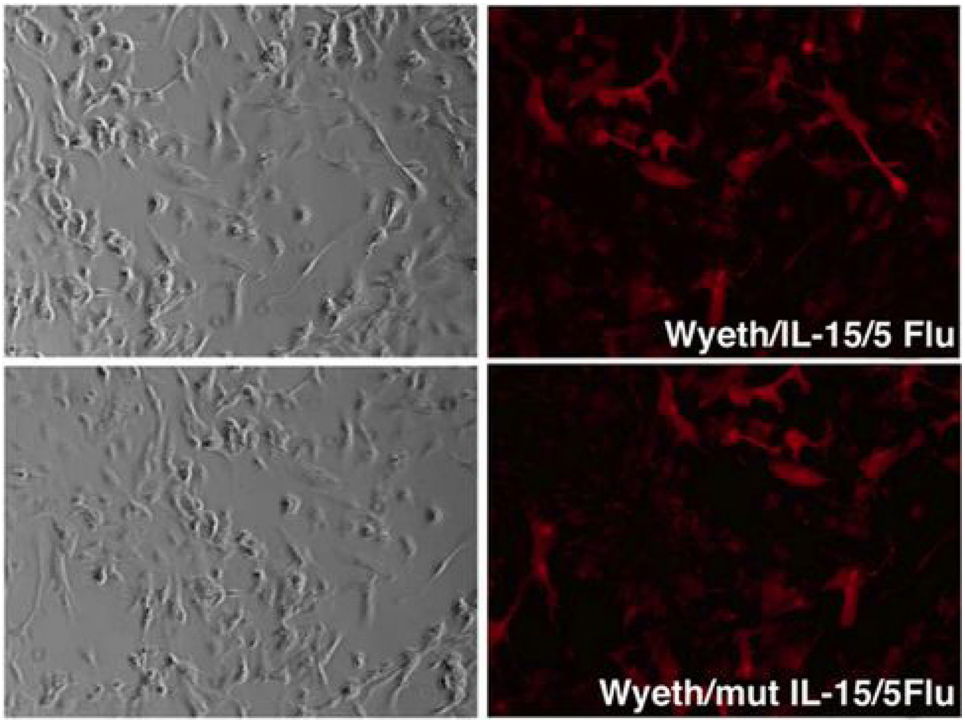


**Figure 2.**
Detection of M2 protein expression by recombinant Wyeth vaccinia viruses by immunofluorescence microscopy. CV-1 cell monolayers infected with Wyeth/IL-15/5Flu or Wyeth/mutIL-15/5Flu reacted with an antibody specific for M2 protein of H5N1 influenza virus. Bound primary antibody was visualized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

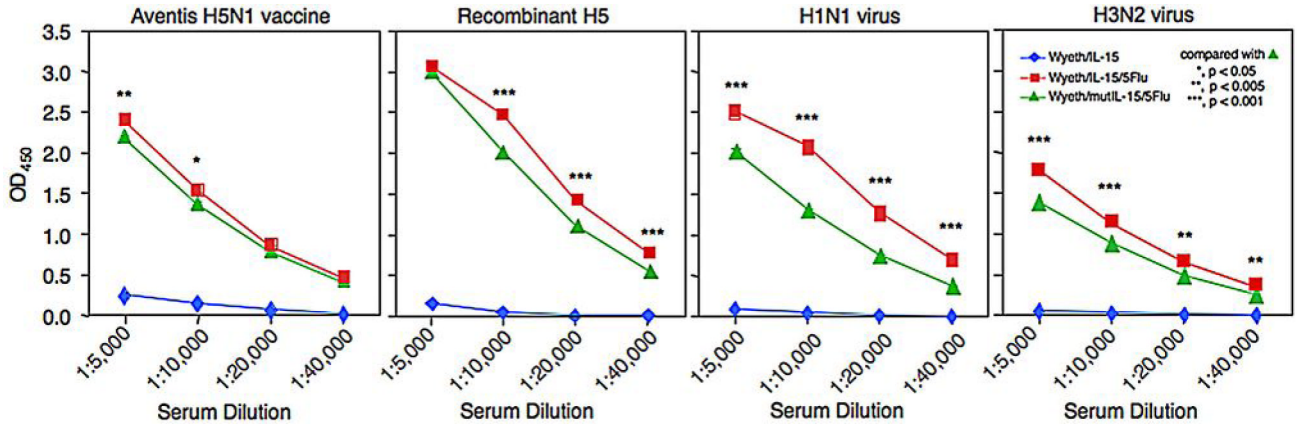


**Figure 3.**

Influenza-specific antibody profiles of mice vaccinated with pentavalent influenza vaccines 4 weeks after booster vaccination. Mice were vaccinated twice four weeks apart subcutaneously with a dose of $1\times10^7$ pfu of virus. Sera collected 4 weeks later and pooled within each group. Wyeth/IL-15 virus vaccinated animals served as a control. Influenza-specific antibodies were detected by an ELISA and the antigen preparation used as the plate bound antigen in each ELISA is indicated on top of each panel.

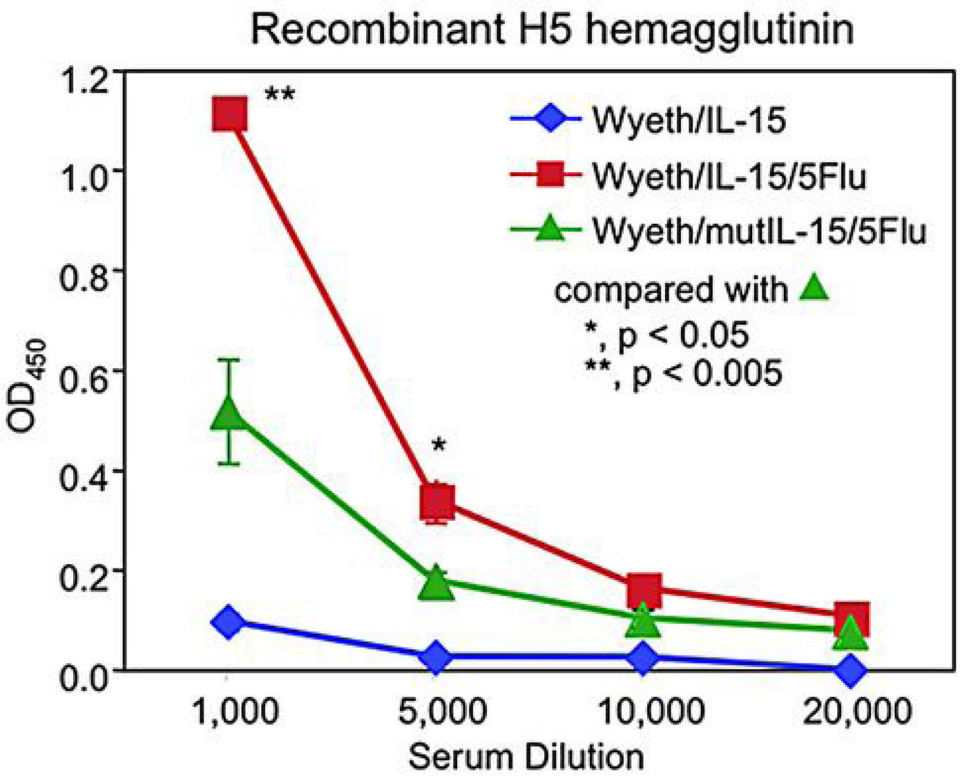


**Figure 4.**

Influenza-specific antibody profiles of mice vaccinated with pentavalent vaccines 14 months after booster vaccination. Mice were vaccinated twice four weeks apart subcutaneously with a dose of $1\times10^7$ pfu of virus. Animals were bled 14 months later and sera separated and pooled within each group. Wyeth/IL-15 virus vaccinated animals served as a control. H5 hemagglutinin-specific antibodies were detected by an ELISA with purified recombinant H5.

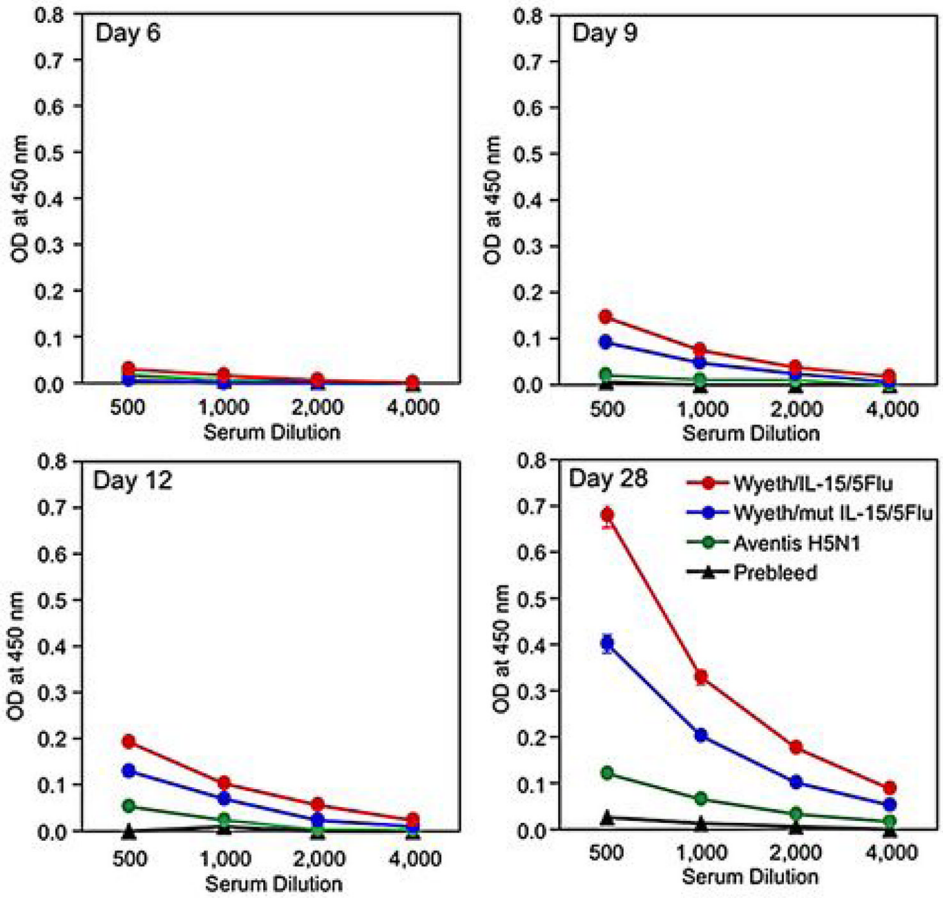


**Figure 5.**

Induction kinetics of influenza-specific antibody responses in vaccinated mice. Mice were given a single dose ($1\times10^7$ pfu of virus) of Wyeth/IL-15/5Flu or Wyeth/mutIL-15/5Flu subcutaneously. Aventis vaccine (rgA/Vietnam/1203/2004) was given intramuscularly (100 microliters of vaccine per mouse injected to the gluteal region). Animals were bled every 3 days post vaccination, sera separated, pooled within each group. H5 hemagglutinin-specific antibodies were detected by an ELISA with recombinant H5 hemagglutinin as the plate-bound antigen. Data shown are selected time points where seroconversions occurred.

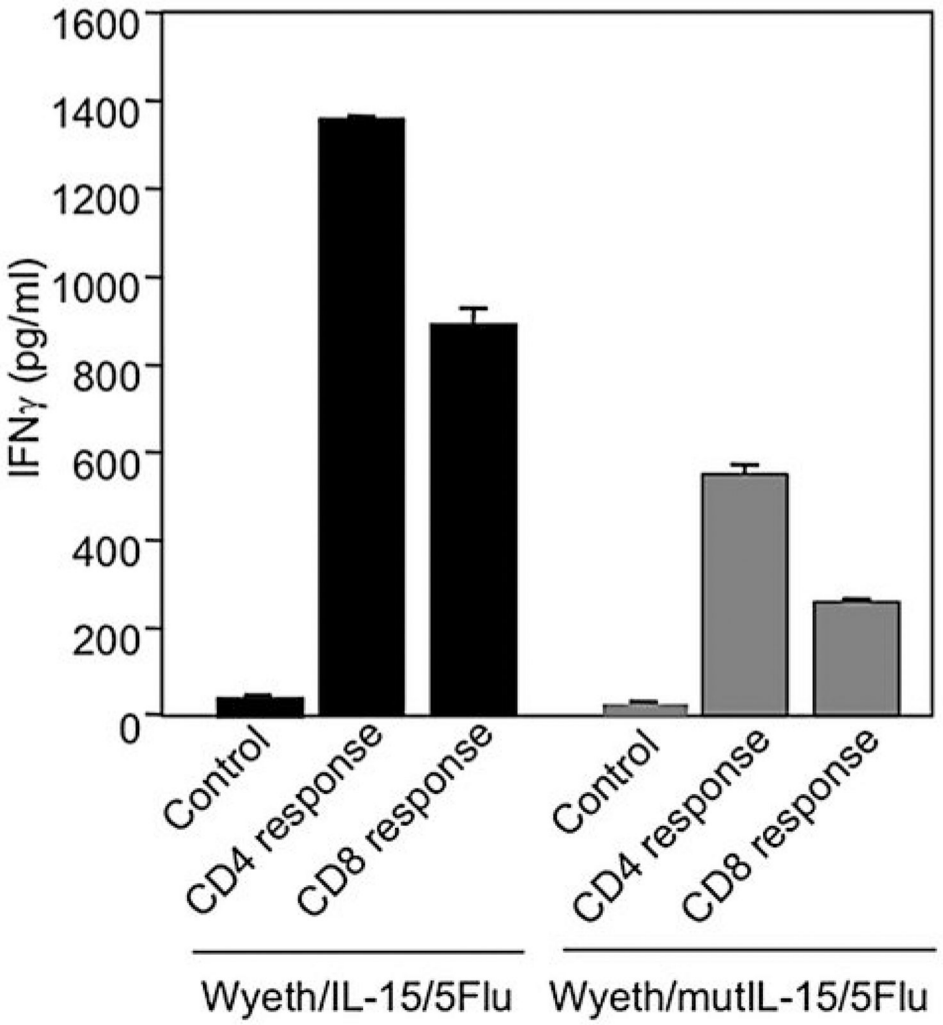


**Figure 6.**

Cellular immune response against influenza antigens in vaccinated mice. Mice were vaccinated twice four weeks apart subcutaneously with a dose of $1\times10^7$ pfu of virus. Three animals from each group were euthanized 8 days later and splenocytes were harvested and pooled. From pooled splenocytes, $CD4^+$ and $CD8^+$ T lymphocytes were purified and then plated at $2\times10^6$ cells per well in triplicate. Age matched, naïve syngeneic splenocytes infected with H1N1 (A/PR/8/34) virus was added to each well after irradiating (3000 rad) at $1\times10^6$ cells per well. Splenocytes ($2\times10^6$ cells per well) from vaccinated animals cultured alone in parallel without irradiated H1N1-infected syngeneic splenocytes served as controls. After a 72-hour period of co-culture, INF-γ levels in the culture supernatants were determined.

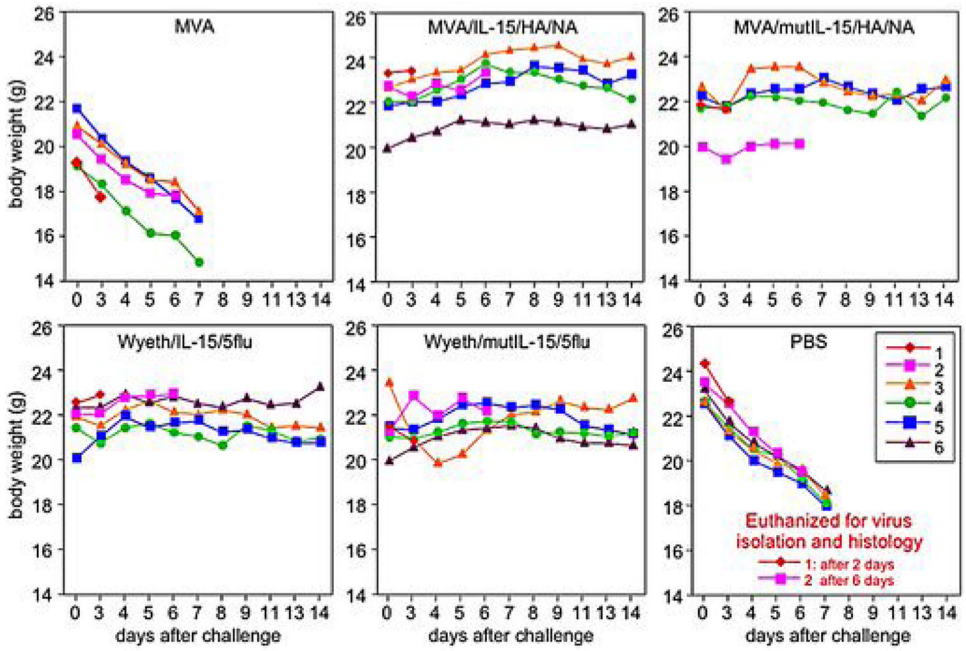


**Figure 7.**

Multivalent vaccinia virus-based H5N1 influenza vaccines protect mice against a heterologous lethal challenge with a HPAI virus. Mice were vaccinated twice four weeks apart subcutaneously with a dose of $1\times10^7$ 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 weeks after the second immunization with a 100 $LD_{50}$ 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 through 6 for identification.

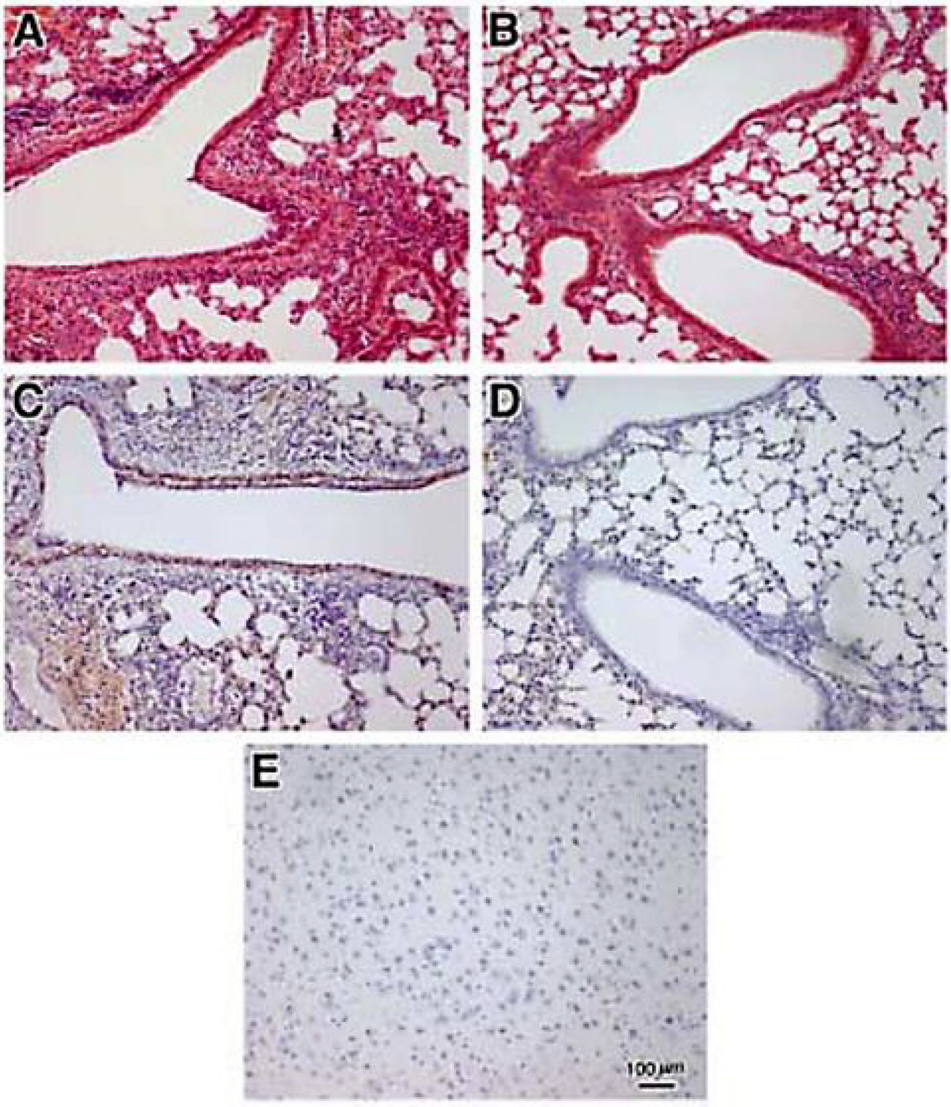


**Figure 8.**

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 post-challenge from each group. Panel A, a representative lung tissue from wild-type MVA vaccinated mice stained with hemotoxylin-eosin. Panel B, a representative lung tissue from an animal vaccinated with MVA/mutIL-15/HA/NA; Panel C, a lung tissue from an animal vaccinated with MVA reacted with monoclonal antibodies to NP and H5 viral antigens by immunohistochemistry. Panel D, a lung tissue from an animal vaccinated with MVA/mutIL-15/HA/NA reacted with the same antibodies used in Panel C; Panel E, a brain tissue section from an animal vaccinated with MVA/mutIL-15/HA/NA reacted with monoclonal antibodies to detect the presence of NP and H5 viral antigens by immunohistochemistry.

**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 * |
|---|---|---|
| Wyeth/IL-15/5Flu | 3500 picog/ml | 900.8 |
| Wyeth/mutIL-15/5Flu | None detected | 2.7 |
| Culture medium | None detected | 1.0 |

* Proliferative index was calculated as the fold-increase in $^{3}$H-thymidine uptake above the media control.

**Table II**

Serum neutralizing antibody titers in vaccinated animals against different clades of H5N1

| Vaccine | clade 1 A/Vietnam/1203/2004 | clade 1 A/Vietnam/1194/2004 | clade 2.2 A/Ck/Indonesia/BL/2003 | clade 2.3 A/Vietnam/30850/2005 |
|---|---|---|---|---|
| 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 |

**Table III**

Virus titers in tissues after challenge and survival of vaccinated mice

| | $TCID_{50}$ virus titer in tissues* | | | % survival** |
|---|---|---|---|---|
| **Vaccine** | **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) |

* Virus titers were determined 3 days post challenge and are expressed as the $\log_{10}$ $TCID_{50}$/ml ± S.D.; NCV-no cuturable virus present

** In parenthesis, number of survivors over total number of animals per group