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Cross-Protective Immunity against Multiple Influenza Virus Subtypes by a Novel Modified Vaccinia Ankara (MVA) Vectored Vaccine in Mice

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Abstract

Development of an influenza vaccine that provides cross-protective immunity remains a challenge. Candidate vaccines based on a recombinant modified vaccinia Ankara (MVA) viral vector expressing antigens from influenza (MVA/Flu) viruses were constructed. A vaccine candidate, designated MVA/HA₁/C13L/NP, that expresses the hemagglutinin from pandemic H1N1 (A/California/04/09) and the nucleoprotein (NP) from highly pathogenic H5N1 (A/Vietnam/1203/04) fused to a secretory signal sequence from vaccinia virus was highly protective. The vaccine elicited strong antibody titers to homologous H1N1 viruses while cross-reactive antibodies to heterologous viruses were not detectable. In mice, this MVA/HA₁/C13L/NP vaccine conferred complete protection against lethal challenge with A/Vietnam/1203/04 (H5N1), A/Norway/3487-2/09 (pandemic H1N1) or A/Influenza/Puerto Rico/8/34 (seasonal H1N1) and partial protection (57.1%) against challenge with seasonal H3N2 virus (A/Aichi/68). The protective efficacy of the vaccine was not affected by pre-existing immunity to vaccinia. Our findings highlight MVA as suitable vector to express multiple influenza antigens that could afford broad cross-protective immunity against multiple subtypes of influenza virus.

Keywords

Influenza; modified vaccinia Ankara; vaccine; cross-protective immunity; safety

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Introduction

Highly communicable, influenza viruses are the cause of yearly epidemics, infecting 5 to 20% of the U.S. population [1]. These infections result in approximately 200,000 hospitalizations and 36,000 deaths per year [2, 3]. Though commonly circulating influenza subtypes cause yearly influenza epidemics, some of these viruses have historically caused pandemic outbreaks as well. The most notorious influenza pandemic was the 1918 Spanish flu, which resulted in the death of an estimated 20–40 million worldwide [4]. The recent pandemic with an H1N1 virus in 2009 resulted in over 18,400 death spanning more than 214 countries worldwide [5]. In contrast to seasonal influenza, most of the serious illnesses caused by the pandemic virus occurred among children, young adults and pregnant women. Concerns remain on how the virus may change as it keeps circulating. The past two decades have seen the emergence of highly virulent avian H5N1 influenza viruses. H5N1 avian influenza first emerged in the human population when the virus crossed the avian to human species barrier in 1997 [6]. These viruses are currently endemic to poultry populations in South East Asia, where they initially emerged [6]. Over the past decade, they have significantly broadened their host and geographic range with a current human mortality rate of approximately 60% [7].

Several types of influenza vaccines are available for use in annual seasonal vaccination, including live attenuated and inactivated vaccines [8–10]. Generation of inactivated vaccines (INV) and cold-adapted, attenuated live viruses (LAV) had been optimized for seasonal flu, but presents several challenges for H5N1 viruses including: 1) continual evolution of the viruses makes predicting a vaccine strain difficult; 2) egg propagation of vaccine stock is hindered due to the high lethality of eggs to H5N1 viruses and, in endemic countries, the poultry that provide them; 3) the 6 to 9 months required to produce new INV or LAV vaccine preparations has proven to be too long to provide protection of large populations in a pandemic setting as it became evident during the recent H1N1 pandemic. The cold adapted, attenuated live virus vaccines elicit both humoral and cellular immunity and broader cross-reactivity. However, they are not recommended in infants, elderly, or immuno-compromised individuals because they can cause pathogenic reactions [11–14]. Thus, the development of new vaccine vectors and production techniques are urgently needed to generate an effective vaccine with broad cross-protective efficacy against various influenza subtypes. The modified vaccinia Ankara (MVA) vector offers several advantages such as; 1) safety, 2) stability, 3) rapid induction of humoral and cellular responses, and 4) multiple routes of inoculation [15–19]. Hessel *et al* [15] described the construction of several MVA recombinant viruses expressing HA proteins of H5N1 viruses from different clades such as A/VN1203, A/Ind/05, A/TT/01/05, A/CE/06 and A/Anhui/05. The vaccine construct expressing the HA from VN1203 provided protection against all these clades in the mouse model. Recently an MVA recombinant virus expressing the HA protein of the pandemic A/CA/09 (H1N1pdm) virus when tested in ferrets was shown to be protective against challenge with the pandemic influenza virus A/Netherlands/602/2009 (H1N1) [17]. While all the above vaccines have their own merit, most of them have been tested against homologous or closely related challenge viruses and provided very limited protection against genetically divergent strains.

This study reports on the efficacy of recombinant MVA vaccines expressing antigens from the pandemic H1N1 virus (A/California/04/09) and the highly pathogenic avian influenza (H5N1) virus A/Vietnam/1203/04.

Materials and Methods

Cells and viruses

Mardin-Darby canine kidney (MDCK) cells obtained from the American Type Culture Collection (ATCC, Manassas, VA) were propagated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics. Stocks of chicken embryo fibroblasts (CEF) were produced as previously described [20, 21]. The CEF were used for propagating modified vaccinia Ankara (MVA) virus obtained from the Centers for Disease Control, Atlanta, GA. Highly pathogenic avian influenza (H5N1) virus (A/Vietnam/1203/04), pandemic H1N1 virus (A/Norway/3487-2/09), seasonal H1N1 virus (A/Puerto Rico/8/34) and H3N2 virus (A/Aichi/2/68) were kindly provided by Dr. Yoshihiro Kawaoka (University of Wisconsin-Madison). The influenza viruses were propagated and titrated in MDCK cells with DMEM that contained 1% bovine serum albumin and 20 mM HEPES and were stored as infectious stocks at -80°C . Viral stock titers were determined by endpoint dilution and recorded as 50% tissue culture infectious dose (TCID_{50}) as previously described [22]. Culture media for H1N1 and H3N2 viruses also included 1 $\mu\text{g/ml}$ of trypsin treated with tosyl phenylalanyl chloromethyl ketone (TPCK). Work with H5N1 influenza virus was conducted in a BSL3+ facility in compliance with the UW Madison Office of Biological Safety.

Construction of plasmids and production of MVA recombinant vaccines

Transfer plasmid pdIIIIGFP encoding green fluorescent protein (kindly provided by Dr. Joanna Shisler, University of Illinois) was used to generate recombinant MVA viruses expressing influenza virus antigens as previously described [23]. Hemagglutinin (HA_1) gene from H1N1pdm (A/California/04/09) and HA_5 gene from H5N1 (A/VN1203/04) virus were synthesized after codon optimization for mammalian expression by GeneScript (Piscataway, NJ). Coding regions of HA_1 and HA_5 proteins were amplified by PCR from the GeneScript constructs using 5' HA_1 ssp/3' HA_1 or 5' HA_5 ssp/3' HA_5 , respectively, (Table 1) to generate appropriate restriction sites and their native secretory signals and then sub-cloned into pdIIIIGFP, generating pdIIIIGFP/ HA_1 and pdIIIIGFP/ HA_5 respectively. An alternative transfer plasmid with a secretory signal, pdIIIIGFP/C13L, was generated by inserting a linker, 5'/3' C13L-ssp, (Table 1) fused with a secretory signal from vaccinia virus (from the N terminus of the C13L vaccinia gene) at the 5' end of the multiple cloning site (MCS) such that antigens could be inserted in frame with the secretory signal. The entire coding region of nucleoprotein (NP) of H5N1 influenza virus (A/Vietnam/1203/04) was amplified by PCR from cDNA clone and then inserted into pdIIIIGFP/C13L to generate the pdIIIIGFP/C13L/NP. A dual transfer vector pdIIIIGFP-d was constructed by inverting the GFP cassette in pdIIIIGFP and then inserting a second promoter/MCS cassette in an inverted orientation to the primary promoter cassette (Figure 1). The coding regions of HA_1 , HA_5 and C13L/NP were sub-cloned from the single expression constructs to generate dual expression constructs pdIIIIGFP/ HA_1 /C13L/NP and pdIIIIGFP/ HA_5 /C13L/NP, respectively (Figure 1). The

recombinant MVA/Flu viruses were generated in CEF cells as described elsewhere [24, 25]. Expressions of recombinant viruses were analyzed by western blot (Supplementary data).

Animal Studies

All mouse studies were conducted at University of Wisconsin-Madison animal facilities and were approved by its Interinstitutional Animal Care and Use Committee (IACUC). Challenge experiments involving H5N1 virus (A/Vietnam/1203/04) were conducted in an ABSL3+ facility in compliance with the University of Wisconsin-Madison Office of Biological Safety.

Vaccine efficacy

A series of experiments were performed to assess the immunogenicity and protective efficacy of MVA/Flu viruses (MVA/HA₁, MVA/HA₅, MVA/C13L/NP, MVA/HA₁/C13L/NP and MVA/HA₅/C13L/NP). Groups (n=10) of five week old BALB/c mice (Harlan, Indianapolis, IN) were vaccinated with 1×10^7 plaque forming unit (pfu) of recombinant MVA/Flu virus contained in 50 μ l of PBS via the intradermal (ID) route. The animals received a single dose or two vaccine doses (28 days apart) and then blood samples were collected 28 and 56 days post-primary vaccination, respectively for serological analysis. At 56 days post-primary vaccination, mice were challenged by intranasal (IN) instillation under isoflurane anesthesia with 100 LD₅₀ of either A/Vietnam/1203/04 (VN/1203) (1×10^4 pfu), A/Norway/3487-2/09 (NW/09) (1.0×10^6 pfu), A/Puerto Rico/8/34 (PR8) (3.99×10^5 pfu) or A/Aichi/2/68 (Aichi/68) (2.5×10^4 pfu) virus contained in 50 μ l of PBS. At day three post-challenge, three mice from each group were euthanized and lung tissues were collected and homogenized using a mechanical homogenizer (MP Biochemicals, Solon, OH). Viral titers in the homogenates were quantified by plaque assay on MDCK cells. Body weight was measured on alternative days and survival rates were recorded daily for 14 days. Mice showing 20% or more of body weight loss were considered to have reached the experimental end point and were humanely euthanized.

Vector immunity

To assess the effect of pre-existing immunity to the vector virus on MVA/Flu vaccines, groups of 4 week old BALB/c mice (n=7) were first injected ID with MVA/GFP (1×10^7 pfu) virus and then vaccinated with the recombinant MVA/FLU vaccine (same route and dose) three months later. Following the initial MVA/GFP inoculation, mice were vaccinated with 2 doses (28 days apart) of either 1×10^7 pfu of MVA/HA5 or MVA/GFP virus followed (28 days post-boost) by a challenge with 100 LD₅₀ of VN/1203 virus. Mice were monitored for morbidity and survival rates recorded over 14 days.

Serology

Blood samples were collected from all animals at different time-points post-vaccination and prior to challenge to determine antibody titers by hemagglutination inhibition (HI) or by microneutralization assays [26]. Briefly, one part of serum sample was added to three parts of receptor destroying enzyme (RDE, Denka Seiken, Tokyo) and incubated at 37°C for 16 hours, then heat-inactivated at 56°C for 1 h. Hemagglutination inhibition (HI) assay was

conducted by mixing 25 μ l of two-fold serial dilutions of RDE treated sera with 8 HA units of virus re-suspended in the same volume of PBS and incubated in 96-well plates for 45 min at room temperature (RT). Following this, 50 μ l of turkey red blood cells (0.5%) were added and plates were incubated at RT for 45 min. HI titers of sera were determined as the highest dilution that displayed hemagglutinin inhibition activity. For microneutralization assay, 50 μ l of two-fold serial dilution of treated serum samples were added to 50 μ l of virus containing 200 TCID₅₀ units and then incubated at 37°C for 1 hour. The virus-serum mixture from each dilution was added to duplicate wells of MDCK cells in 96-well plates, incubated at 37°C for 72 hours, fixed and stained with 10% crystal violet in 10% formalin to determine the TCID. The titer was defined as the serum dilution resulting in complete neutralization of the virus.

T cell responses by Flow cytometric analysis

Three weeks following a single dose of MVA/HA₁/C13L/NP, MVA/HA₅/C13L/NP or MVA/GFP, immunized mice (n=3) were euthanized and spleens were harvested. Splenocytes from individual animals were suspended in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 I.U./ml penicillin, 100 μ g/mL streptomycin and 0.14mM β -mecaptoethanol. Red blood cells were lysed with 1x BD Pharm LyseTM buffer. Following washing with RPMI medium, cells were resuspended in the same medium and 1×10^6 splenocytes were surface stained with anti-mouse CD4 FITC (RM4-5) and anti-mouse CD8a PerCP (53-6.7) mAbs.

To study intracellular cytokine responses, 1×10^6 splenocytes were plated to a 96-well flat-bottom plate and stimulated with inactivated VN/1203 whole virus (1 μ g/ml) or infectious influenza viruses (NW/09 or Aichi/68) at an MOI of 1 and an MOI of 0.1 for PR8 in 200 μ l total volume for 21 hours. Bredfeldin A (BD GolgiPlug) was added at a final concentration of 1 μ g/ml for the last 5 hr of incubation to block protein transport. Cells were stained intracellularly for IFN- γ APC (XMG1.2) and IL-2 PE (JES6-5H4) after surface staining for CD4 and CD8a. All antibodies were from BD Bioscience except where it was noted. The samples were acquired on BD FACSCalibur and analyzed with FlowJo v7.6.5 (Tree Star). The cytokine background from medium-treated groups was subtracted from each sample. The frequency of cytokine-positive T cells was presented as the percentage of gated CD4+ or CD8+ T cells.

Statistical analysis

One way ANOVA was used to evaluate the vaccine group effects on pre-boost and pre-challenge antibody titers. Survival analyses were performed to assess vaccine effectiveness against challenge viruses; reported *P*-values are from Fisher's exact test. Probability values < 0.05 were considered significant using the GraphPad Prism 5 software (La Jolla, CA) for all statistical analyses.

Results

Efficacy of a single dose of MVA/Flu vaccine against homologous challenge

Antibody titers from immunized mice were measured by hemagglutination Inhibition (HI) assay against A/Norway/3487-2/09(NW/09) or A/Vietnam/1203/04 (VN/1203), respectively and survival rates following challenge recorded. The vaccines elicited significantly higher ($P < 0.05$) HI titers (Figure 2A and B) compared to the control virus (MVA/GFP) and conferred complete protection against homologous challenge with NW/09 or VN/1203, respectively (Figure 2C and D). Although one out of three mice vaccinated with a single dose of MVA/HA₁ or MVA/HA₅ showed high titers of virus in their lungs on day 3 post challenge, the overall viral loads in the lungs of immunized mice were significantly lower ($p < 0.05$) compared to those from MVA/GFP immunized mice (Figure 2E and F). Furthermore, mice receiving a prime and boost immunization with MVA/Flu vaccine in subsequent studies, cleared the virus from their lungs three days post-challenge (data not shown). Mice in the control group lost weight and were humanely euthanized when they reached 20% weight loss. No significant ($P > 0.05$) weight losses were recorded in animals that received any of the MVA/Flu vaccines (MVA/HA₁ or MVA/HA₅) upon challenge with NW/09 or VN/1203, respectively (Figure 2G and H). In an ancillary long-term immunity study, mice were immunized with a single dose of MVA/HA₅ and seven months later they were challenged with a lethal dose of VN/1203 (Figure 3A and B). All vaccinated animals were protected while control animals succumbed to challenge with a median survival day of seven (Figure 3B).

Cross-protective immunity of MVA/Flu vaccines

In our studies we also tested the cross-protective efficacy of MVA/Flu vaccines expressing both HA and NP proteins (MVA/HA₁/C13L/NP and MVA/HA₅/C13L/NP) against IN challenge with 100 LD₅₀ of the H5N1 strain A/Vietnam/1203/04 (VN/1203) (1×10^4 pfu), the H1N1 pandemic strain A/Norway/3487-2/09 (NW/09) (1.0×10^6 pfu), the H1N1 seasonal strain A/Puerto Rico/8/34 (PR8) (3.99×10^5 pfu) or the H3N2 seasonal strain A/Aichi/2/68 (Aichi/68) (2.5×10^4 pfu) virus. Following prime and booster vaccinations, strong antibody titers were induced against the homologous virus strains; however no cross-reactive antibodies were detected against heterologous viruses (Table 2). In particular, the MVA/HA₁/C13L/NP vaccine elicited strong HI antibody titers to NW/09 virus but failed to induce any antibody responses to the heterologous strains PR8, VN/1203 or Aichi/68 viruses (Table 2). Despite the lack of cross-reactivity in HA titers, complete protection against challenge with PR8 or VN/1203 was observed (Table 2). In addition, the MVA/HA₁/C13L/NP vaccine conferred partial protection (57.1%) against challenge with Aichi/68 virus (Table 2). In the case of the MVA/HA₅/C13L/NP virus, it did not induce any detectable antibody responses to any of the H1N1 viruses (NW/09 and PR8) or H3N2 (Aichi/68) virus. However, it did protect immunized mice against challenge with the H1N1 PR8 strain (Table 2). Mice immunized with MVA/HA₁/C13L/NP vaccine had no significant weight loss following challenge with the H5N1 VN/1203 strain whereas those immunized with the MVA/HA₅/C13L/NP virus had significant weight loss upon challenge with H1N1 pandemic NW/09 strain (Figure 4A and B). Mice in the control group persistently lost weight upon challenge with VN/1203, NW/09, PR8 or Aichi/68 virus (Figure 4A, B, C and D).

Consistent with the cross-protection survival data, mice immunized with MVA/HA₅/C13L/NP showed significant weight loss ($P < 0.05$) compared to mice immunized with MVA/HA₁/C13L/NP upon challenge with the H3N2 Aichi/68 virus (Figure 4D); however no significant differences in weight were recorded in mice immunized with MVA/HA₁/C13L/NP or MVA/HA₅/C13L/NP vaccines following challenge with seasonal H1N1 PR8 virus (Figure 4C). Taken together, these data indicate that the MVA/HA₁/C13L/NP vaccine provides the broadest cross-protective immunity, conferring complete protection from all three group 1 A strains and partial protection against the group 2 H3N2 strain. Interestingly, the MVA/HA₅/C13L/NP strain completely protected against heterologous challenge with the seasonal H1N1 strain, but not the pandemic H1N1 strain, nor the H3N2 strain.

T cell responses

The recombinant MVA vaccines elicited high numbers of activated CD4⁺ and CD8⁺ T cells in mice (Figure 5A). Mice immunized with MVA/HA₁/C13L/NP or MVA/HA₅/C13L/NP elicited varying levels of interferon-gamma (IFN- γ) responses from CD4⁺ or CD8⁺ T cells upon stimulation with the test viruses (Figure 5B and C). Stronger IFN- γ responses were elicited by CD8⁺ compared to the CD4⁺ T cells. Interestingly very low frequency of IFN- γ responses were recorded from immunized mice upon stimulation with PR8. No detectable antigen specific IFN- γ responses were recorded in the control mice (MVA/GFP) upon stimulation with any of the test viruses. Interleukin-2 (IL-2) responses were detectable in CD4⁺ T cells but not in CD8⁺ T cells (data not shown).

Vector immunity

Pre-exposure of mice to MVA significantly ($P < 0.05$) affected the neutralizing antibody titers elicited post-priming with MVA/HA₅ vaccine. However this effect was reversed following a booster vaccination with the same virus (Figure 6A). Moreover, pre-existing immunity to MVA did not have any significant impact on the protective efficacy of the vaccine after prime and booster immunizations (Figure 6B).

Discussion

In the present study, we evaluated the potential of several recombinant MVA-vectored vaccines expressing antigens from influenza viruses to provide protection against various influenza subtypes. We also assessed the advantages of combining antigens from different influenza subtypes on cross protective efficacy of a recombinant vaccine.

Vaccine viruses expressing hemagglutinin from H1N1 (MVA/HA₁) or H5N1 (MVA/HA₅) conferred complete protection against homologous challenge with A/Norway/3487-2/09 (NW/09) or A/Vietnam/1203/04 (VN/1203) respectively, confirming previous reports [15, 17, 27, 28]. In addition, a single dose of the MVA/HA₅ vaccine afforded long-term protection (7 months) against challenge with a lethal dose of the highly pathogenic H5N1 avian influenza virus (VN/1203). This is the first report of long-term immunity study using an MVA-vectored vaccine.

The continued evolution of influenza viruses in diverse host populations always poses global health challenges necessitating development of vaccines with broad cross-protective

immunity against subtypes of influenza virus. Recent reports have documented the important role of cross-protective immunity due to prior exposure to seasonal influenza viruses on the immune responses following exposure to H5 and H1 viruses [29–34]. Based on these observations and due to the potential for a pandemic H5N1 outbreak, we developed MVA/Flu vaccines expressing the nucleoprotein from the H5N1 VN/1203 strain and the hemagglutinin from either the H1N1 pandemic CA/09 strain (MVA/HA₁/C13L/NP) or the H5N1 VN/1203 strain (MVA/HA₅/C13L/NP). Testing these recombinant viruses in mice elicited strong humoral responses against homologous viruses but no cross-reactive antibodies to heterologous viruses. Although, the MVA/HA₅/C13L/NP vaccine did not elicit any detectable cross-reactive HI antibodies against PR8, it did confer complete protection against a lethal challenge dose of PR8 virus. In the absence of cross-protective antibodies, the MVA/HA₅/C13L/NP vaccine did not protect against challenge with NW/09 or Aichi/68 viruses. It is not surprising that the protective efficacy of MVA/HA₅/C13L/NP vaccine against challenge with PR8 and NW/09 was significantly different, since the pandemic H1N1 viruses are antigenically different from the seasonal H1N1 viruses [30, 31]. The ability of MVA/HA₅/C13L/NP vaccine to confer complete protection against challenge with PR8 suggests a possible role for cellular immunity in protection. The nucleoprotein (NP) is a conserved protein in influenza viruses known to possess cytotoxic T lymphocyte (CTL) epitopes [35–37]. In addition to the NP CTL responses, the HA also possesses CTL epitopes which could have also played a role in cross-protection [15, 38]. Although all immunized mice upon challenge with PR8 were completely protected, analyses of T cell responses by flow cytometry demonstrated the induction of IFN- γ in CD4+ or CD8+ T cells. Therefore complete protection can be attributed to T cells and/or non-neutralizing antibodies that could contribute to antibody dependent cytotoxicity in the clearance of infected cells. Thorough analyses of cellular immunity are currently underway for further characterization of CTL responses elicited by the vaccine candidates.

The MVA/HA₁/C13L/NP vaccine did not elicit humoral responses to VN/1203, PR8 or Aichi/68 viruses. However, all immunized mice were fully protected against challenge with the H5N1 VN/1203 strain or the seasonal H1N1 PR8 strain, respectively and a partial protection (57.1%) against challenge with the H3N2 Aichi/68 virus. In general the MVA/HA₁/C13L/NP vaccine was superior to the MVA/HA₅/C13L/NP construct in terms of cross-protective efficacy. In addition to complete protection against homologous virus, the MVA/HA₁/C13L/NP vaccine conferred complete protection against two antigenically different viruses (VN/1203 and PR8) whereas the MVA/HA₅/C13L/NP vaccine could completely protect against a challenge with only PR8. The MVA/HA₁/C13L/NP vaccine also afforded significantly better protection against challenge with Aichi/68 compared to the MVA/HA₅/C13L/NP vaccine. The difference in cross-protection by the two vectors is surprising, since both share common NP sequences and neither induced potent cross-reactive antibodies detectable in HI assays. Either undetectable humoral responses or cellular responses to HA₁ sequences may contribute to the cross-protection. Our data further showed that the presence of pre-existing anti-vector immunity may not be a major concern for the efficacy of MVA-vectored vaccines, confirming previous reports [39–41].

The ability of the MVA/HA₁/C13L/NP vaccine to confer complete protection against multiple subtypes of influenza A viruses as well as favorable properties of the MVA

backbone, such as facile genetic manipulation, rapid and easy production at large scale under BSL-1 conditions, and prolonged stability to allow stockpiling of the vaccine, makes MVA/HA₁/C13L/NP virus an attractive and promising universal vaccine candidate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

1. We have developed an influenza vaccine with cross protective immunity
2. The vaccine fully protects against seasonal and pandemic H1N1 influenza viruses in mice
3. Vaccine also fully protects against H5N1 influenza virus
4. Vaccine has the potential to protect against small pox virus

Figure 1.

Schematic representation of recombinant plasmid construction

Expression cassettes were generated by PCR for each of the influenza virus hemagglutinin antigens, HA₁ and HA₅ as described in the materials and methods. The cassettes were cloned into the pIIIIGFP vector and the resulting plasmids were designated as pIIIIGFP/HA₁ and pIIIIGFP/HA₅. The coding regions of HA₁, HA₅ and C13L/NP were sub-cloned from the single expression constructs to generate dual expression constructs pIIIIGFP/HA₁/C13L/NP and pIIIIGFP/HA₅/C13L/NP. Homologous recombination into MVA was successfully completed and recombinant MVA/Flu viruses were identified by GFP expression. HA, NP and C13L are represented with orange, green and pink color codings. MCS1 and MCS2-multiple cloning sites; Flanks1 and Flanks2-open box, GFP-stripped box; dSP - orthopoxvirus secretory signals, either p11 (gfp) or synthetic early/late promoter (MCS1/2) and delIII - MVA deletion region III.

Figure 2.

Vaccine efficacy against homologous challenge

Mice (n=10) were immunized with MVA/HA₁ or MVA/HA₅ and then challenged against pandemic H1N1 (NW/09) (A, C, E, G) or H5N1 (VN/1203) (B, D, F, H). Fifty-six days following primary immunization antibody titers were determined by HI assay (A, B). Mice were challenged 56 days post primary immunization and survival rates were recorded (C, D). Three days post-challenge lung viral load were determined (E, F) and clinical scores (weight) measured over 14 days post-challenge (G, H).

Figure 3.

Long-term vaccine efficacy against homologous challenge

Mice (n=7) were immunized with MVA/HA₅ and then challenged against H5N1 (VN/1203).

Seven Twenty eight weeks (7 months) following immunization with a single dose of the vaccine and mice were challenged and then weight changes (A) and survival rates (B) were recorded over 14 days post-challenge.

Figure 4.

Cross protective efficacy of MVA/Flu vaccines

Mean weight of immunized mice (n=7) following challenge with A (VN/1203), B (NW/09), C (PR8) or D (Aichi/68).

Figure 5.

T cell responses to immunization

Three weeks following a single dose of MVA/HA1/C13L/NP, MVA/HA5/C13L/NP or MVA/GFP, 1×10^6 splenocytes from immunized mice were stimulated with inactivated VN/1203 whole virus (1 μ g/ml) or infectious influenza viruses (NW/09 or Aichi/68) at an MOI of 1 and an MOI of 0.1 for PR8. Samples were analyzed for T cell responses by flow cytometry.

Figure 6.

Effects of vector immunity on vaccine efficacy

Cohorts of mice ($n=7$) were first injected with MVA vector and then received two doses of MVA/Flu vaccine three months later. Following prime and booster vaccinations, antibody titers were measured by microneutralization (A) and twenty eight days following the booster vaccination, mice were challenged with VN/1203 and survival rates were recorded (B).

Table 1

Sequence of primers used for amplification of influenza virus proteins

Primer	PCR Primer Sequence ^a	Restriction Enzyme
5' HA ₅ ssp	5'-tctcCCCGGGatggagaaaatagtgctctttttgcaatagtcagcttggtaaaagtgatcagattgcattgggtacat-3'	XmaI
3' HA ₅	5'-ggcGGATCCttaaatgcaaatctgcattgt-3'	NgomIV
5' HA ₁ ssp	5'-gtgaCCCGGGatgaaggccatcctggtcgtgctgtacacctcgccaccgccaacgccgacacactgtgtatcgggtat-3'	XmaI
3' HA ₁	5'-gatccGCTAGCtcagatacagat-3'	NheI
5' NP	5'-gtgaGCCGGCgcgtctcaaggcaccaaa-3'	NgomIV
3' NP	5'-gcGAATTCttaattgcatactcctctgcattgt-3'	EcoRI
5' C13L-ssp	5'-CCGGGatgatgatatacggattaatagcgtgtcttatattcgtgacttcacatcgctagccaGCCGGCG-3'	XmaI, NgomIV, NheI
3' C13L-ssp	5'-CTAGCGCCGGCtggactagcgtggatgaagtcacgaatataagacacgctattaatccgtatatcatcatC-3'	XmaI, NgomIV, NheI

^a Restriction enzyme sites are capitalized.

Table 2

Cross-protective immunity of MVA/Flu vaccines

Vaccines	Challenge virus									
	H5N1 (VN/1203)		H1N1pdm (NW/09pdm)		H1N1 seasonal (PR8)		H3N2 seasonal (Aichi/68)			
	Post-Boost Titer (GMT)	% Survival	Post-Boost Titer (GMT)	% Survival	Post-Boost Titer (GMT)	% Survival	Post-Boost Titer (GMT)	% Survival	Post-Boost Titer (GMT)	% Survival
MVA/HA ₁ /C13L/NP	<20 ^a	100	373	100	<20	100	<20	100	<20	57.1
MVA/HA ₃ /C13L/NP	149	100	<20	28.6	<20	100	<20	100	<20	14.3
MVA/GFP	<20	0	<20	28.6	<20	14.3	<20		<20	28.6

^aBelow limit of detection. Hemagglutination inhibition antibody titers are presented as geometric mean.