(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
9 August 2012 (09.08.2012)

WIPO | PCT

(10) International Publication Number
WO 2012/106231 A2

(51) **International Patent Classification**:
A61K 39/145 (2006.01)

(21) **International Application Number**:
PCT/US2012/023085

(22) **International Filing Date**:
30 January 2012 (30.01.2012)

(25) **Filing Language**: English

(26) **Publication Language**: English

(30) **Priority Data**:
61/438,024 31 January 2011 (31.01.2011) US

(71) **Applicants** *(for all designated States except US)*: **BAXTER INTERNATIONAL INC.** [US/US]; One Baxter Parkway, Deerfield, IL 60015 (US). **BAXTER HEALTHCARE S.A.** [CH/CH]; Thurgauerstrasse 130, CH-8152 Glattpark (Opfikon) (CH).

(72) **Inventors; and**

(75) **Inventors/Applicants** *(for US only)*: **FALKNER, Falko-Günter** [DE/AT]; Neusiedlzeile 76A, A-2304 Orth/Donau (AT). **SCHÄFER, Birgit** [DE/AT]; Grünentorgasse 24/7, A-1090 Wien (AT). **HESSEL, Annett** [AT/AT]; Hauptstrasse 24, A-2304 Orth/Donau (AT). **BARRETT, P., Noel** [IE/AT]; Steinwandgasse 6A, A-3400 Klosterneuburg/Weidling (AT). **KREIL, Thomas, R.** [AT/AT]; Ziegelofengasse 93-95, A-3400 Klosterneuburg (AT). **EHRLICH, Hartmut** [DE/AT]; Bienenweg 1, A-1170 Wien (AT).

(74) **Agent**: **NOLAND, Greta, E.**; Marshall, Gerstein & Borun LLP, 233 S. Wacker Drive, 6300 Willis Tower, Chicago, IL 60606-6357 (US).

(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

— *with sequence listing part of description (Rule 5.2(a))*

(54) **Title**: RECOMBINANT VIRAL VECTORS AND METHODS FOR INDUCING A HETEROSUBTYPIC IMMUNE RESPONSE TO INFLUENZA A VIRUSES

(57) **Abstract**: The present invention relates to recombinant viral vectors and methods of using the recombinant viral vectors to induce an immune response to influenza A viruses. The invention provides recombinant viral vectors based, for example, on the non-replicating modified vaccinia virus Ankara. When administered according to methods of the invention, the recombinant viral vectors are designed to be cross-protective and induce heterosubtypic immunity to influenza A viruses.

WO 2012/106231 A2

RECOMBINANT VIRAL VECTORS AND METHODS FOR INDUCING A HETEROSUBTYPIC IMMUNE RESPONSE TO INFLUENZA A VIRUSES

FIELD OF THE INVENTION

[0001] The present invention relates to recombinant viral vectors and methods of using the recombinant viral vectors to induce an immune response to influenza A viruses. The invention provides recombinant viral vectors based, for example, on the non-replicating modified vaccinia virus Ankara. When administered according to methods of the invention, the recombinant viral vectors are designed to be cross-protective and induce heterosubtypic immunity to influenza A viruses.

BACKGROUND OF THE INVENTION

[0002] Human influenza or "the flu" is a respiratory disease that is caused by influenza A and B viruses. Epidemics of influenza cause significant illness and death worldwide each year, and vaccination is the most straightforward strategy to prevent infection and disease. Traditional influenza vaccines expose the recipient to influenza virus proteins causing the recipient to mount an immune response to the proteins. Proteins (or polypeptides) used in vaccines are commonly called "antigens." The commonly used seasonal influenza vaccines are based on the major antigen of the viruses, the hemagglutinin (HA). There are numerous influenza A subtypes having different HA antigens. Influenza A subtypes are divided and classified based on the HA and neuraminidase (NA) proteins that are expressed by the viruses. The influenza A subtype nomenclature is based on the HA subtype (of the sixteen different HA genes known in the art) and the NA subtype (of the nine different NA genes known in the art). Exemplary subtypes, include, but are not limited to, H5N1, H1N1 and H3N2. There are also variants of the influenza A subtypes which are referred to as "strains." For example, the virus A/VietNam/1203/2004 is an influenza A virus, subtype H5N1, with a strain name A/VietNam/1203/2004.

[0003] Protection from the seasonal vaccines based on the HA is strain-specific and new strains emerge constantly, so the classical influenza vaccines have to be re-formulated each year in an attempt to match the currently circulating strains. See, Lambert and Fauci 2010. It is

therefore highly desirable for next generation vaccines to be cross-protective and induce heterosubtypic immunity, *i.e.*, vaccines against one subtype that protect or partially protect against challenge infection with influenza A of different subtypes.

[0004] The current 'universal vaccines' (*i.e.*, vaccines designed to elicit heterosubtypic immunity) that are under development are mainly based on the more conserved internal influenza virus genes including the influenza matrix proteins (M1 and M2) (Schotsaert *et al.* 2009), the nucleoprotein (NP) and conserved parts of the HA (Bommakanti *et al.* 2010; Steel *et al.* 2010). The polymerase proteins PA, PB1 and PB2 also induce substantial T cell responses and may be also relevant targets (Assarsson *et al.* 2008; Greenbaum *et al.* 2009; Lee *et al.* 2008).

[0005] Next generation influenza vaccines currently under development include recombinant proteins, synthetic peptides, virus-like particles (VLPs), DNA-based vaccines and viral vector vaccines (Lambert and Fauci, *supra*). The advantage of using live viral vectors is their known property to induce high levels of cellular immunity, in particular CD8 T cells. Among the most promising viral vectors are vaccinia virus-based live vaccines (Rimmelzwaan and Sutter 2009) and adenovirus-based vectors (Hoelscher *et al.* 2006; Hoelscher *et al.* 2007; Price *et al.* 2010; Zhou *et al.* 2010). Single-dose mucosal immunization using an adenovirus construct expressing NP and M2, for instance, provided protection from virulent H5N1, H3N2 and H1N1 viruses (Price et al, supra). In a further study (Price *et al.* 2009), DNA vaccination with nucleoprotein (NP) and matrix 2 (M2) plasmids followed by boosting with antigen-matched recombinant adenovirus (rAd) provided robust protection against virulent H1N1 and H5N1 challenges in mice and ferrets.

[0006] Recombinant vaccines based on modified vaccinia virus Ankara (MVA) have been used in many non-clinical and clinical studies. MVA has proven to be exceptionally safe. No significant side effects have been obtained when MVA was administered to more than 120,000 human patients in the context of the smallpox eradication. Due to a block in virion morphogenesis the highly attenuated vaccinia virus strain fails to productively replicate in human and most other mammalian cells. Nevertheless, the ability to express viral and foreign genes in the early and late stage is retained. These characteristics make MVA a promising live vaccine vector that induces humoral and cellular immune responses and that exhibits a high safety profile.

[0007] U.S. Patent Nos. 6,998,252; 7,015,024; 7,045,136 and 7,045,313 relate to recombinant poxviruses, such as vaccinia.

[0008] MVA-based vaccines have been used in clinical studies, for instance, against HIV, tuberculosis, malaria and cancer. In all of these studies, at least two doses were used. The human dose of an MVA-based vaccine was $5x10^7$ to $5x10^8$ PFU as applied in clinical trials (Brookes *et al.* 2008; Cebere *et al.* 2006;Tykodi and Thompson 2008;).

[0009] MVA has been used recently as a vector in pandemic H5N1 (Kreijtz *et al.* 2008; Kreijtz *et al.* PLoS One 2009; Kreijtz *et al.* Vaccine 2009; Kreijtz *et al.* J. Infect. Dis. 2009; Kreijtz *et al.* 2007; Mayrhofer *et al.*, 2009; Poon *et al.* 2009) and H1N1 (Hessel *et al.* 2010; Kreijtz *et al.*, J. Infect. Dis. 2009) influenza research. An MVA-based vaccine expressing NP and M1 is currently being tested in an ongoing clinical trial (Berthoud *et al. 2011).*

[0010] Thus, there remains a need in the art for a more broadly protective influenza vaccine.

DETAILED DESCRIPTION

[0011] The present invention provides recombinant viruses (also referred to as recombinant viral vectors herein) useful for generating a heterosubtypic immune response to influenza A viruses. The recombinant viruses are recombinant vaccinia viruses, such as recombinant MVA or other non-replicating or replicating vaccinia virus known in the art. Non-replicating vaccinia viruses include, but are not limited to, defective vaccinia Lister (dVV), MVA-575 (ECACC V00120707), MVA-BN (ECACC V00083008), MVA-F6 and MVA-M4 (Antoine *et al.* 1998). In some embodiments, the recombinant viruses encode a fusion protein (hlHA/M2e) comprising an influenza A hemagglutinin deletion mutant "headless HA" (hlHA) with at least one influenza A M2 external domain (M2e) insert; an hlHA/M2e fusion protein and an influenza A nucleoprotein (NP); or an hlHA and NP. The recombinant viruses of the invention may further encode an influenza A matrix protein 1 (M1) and/or an influenza A polymerase PB1. When administered according to methods of the invention, the recombinant viruses are cross-protective and induce heterosubtypic humoral and cellular immune responses (including CD8 and CD4 T cell responses). The recombinant viruses are therefore contemplated to be useful as universal influenza A vaccines in humans.

[0012] In some embodiments, the hlHA amino acid sequence encoded by an open reading frame in recombinant viruses of the invention may be, for example, the hlHA amino acid sequence set out in SEQ ID NO: 15 (based on A/VietNam/1203/2004 H5N1 HA NCBI Genbank AAW80717 which is SEQ ID NO: 3). The hlHA of SEQ ID NO: 15 comprises a signal sequence, the HA1 residues 17-58 of SEQ ID NO: 3, a linker peptide of four glycines, the HA1 residues 290-343 of SEQ ID NO: 3 and the HA2 stalk region residues 344-568 of SEQ ID NO: 3.

[0013] In some embodiments, the hlHA/M2e fusion protein amino acid sequence encoded by an open reading frame in recombinant viruses of the invention may be, for example, the hlHA/M2e fusion protein amino acid sequence set out in SEQ ID NO: 2. The fusion protein of SEQ ID NO: 2 comprises a signal sequence, the HA1 residues 17-58 of SEQ ID NO: 3, a linker peptide of three glycines (SEQ ID NO: 4), the M2e of H5N1 (SEQ ID NO: 5 based on A/VietNam/1203/2004 H5N1 NCBI Genbank ABP35634), a six-amino acid linker GSAGSA (SEQ ID NO: 9), the M2e of H1N1 (equivalent to H2N2 and H3N2) (SEQ ID NO: 6 based on A/New York/3315/2009 H1N1 NCBI Genbank ACZ05592), a six-amino acid linker GSAGSA (SEQ ID NO: 9), the M2e of H9N2 (SEQ ID NO: 7 based on A/chicken/Korea/SH0913/2009 H9N2 NCBI Genbank ADQ43641), a six-amino acid linker GSAGSA (SEQ ID NO: 9), the M2e of H7N2 (SEQ ID NO: 8 based on A/New York/107/2003 H7N2 NCBI Genbank ACC55276), a linker peptide of three glycines (SEQ ID NO: 4), the HA1 residues 290-343 of SEQ ID NO: 3 and the HA2 region residues 344-568 of SEQ ID NO: 3.

[0014] In some embodiments, the h1HA/M2e fusion protein may comprise one, two, three or four of the M2e polypeptides of SEQ ID NOs: 5, 6, 7 and 8. The h1HA/M2e fusion protein may comprise an influenza A M2e polypeptide other than an M2e polypeptide of SEQ ID NOs: 5, 6, 7, and 8.

[0015] In some embodiments, the NP amino acid sequence encoded by an open reading frame in recombinant viruses of the invention may be, for example, the NP amino acid sequence set out in SEQ ID NO: 13 (based on A/VietNam/1203/2004 H5N1 NP NCBI Genbank AAW80720). In some embodiments, the M1 amino acid sequence encoded by an open reading frame in recombinant viruses of the invention may be, for example, the M1 amino acid sequence set out in SEQ ID NO: 11 (based on A/VietNam/1203/2004 H5N1 M1 Genbank AAW80726). In some

embodiments, the PB1 amino acid sequence encoded by an open reading frame in recombinant viruses of the invention may be, for example, the PB1 amino acid sequence set out in SEQ ID NO: 17 (based on A/VietNam/1203/2004 H5N1 PB1 Genbank AAW80711).

[0016] The invention contemplates that polypeptides encoded by an open reading frame in a recombinant virus may vary in sequence from SEQ ID NO: 2, 5, 6, 7, 8, 11, 13, 15 and/or 17 if the polypeptides retain the ability to induce a protective immune response when the recombinant virus is administered to an individual. In these embodiments, the polypeptide may be about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 95%, about 97%, about 98% or about 99% identical to SEQ ID NO: 2, 5, 6, 7, 8, 11, 13, 15 and/or 17 .

[0017] In other embodiments, hlHA/M2e fusion proteins, hlHA polypeptides and NP polypeptides encoded by recombinant viruses of the invention may be based on the same or different influenza A subtypes including, but not limited to, any combination of H1 to H16 and N1 to N9 (including H1N1, H2N1, H3N1, H4N1, H5N1, H6N1, H7N1, H8N1, H9N1, H10N1, H11N1, H12N1, H13N1, H14N1, H15N1, H16N1; H1N2, H2N2, H3N2, H4N2, H5N2, H6N2, H7N2, H8N2, H9N2, H10N2, H11N2, H12N2, H13N2, H14N2, H15N2, H16N2; H1N3, H2N3, H3N3, H4N3, H5N3, H6N3, H7N3, H8N3, H9N3, H10N3, H11N3, H12N3, H13N3, H14N3, H15N3, H16N3; H1N4, H2N4, H3N4, H4N4, H5N4, H6N4, H7N4, H8N4, H9N4, H10N4, H11N4, H12N4, H13N4, H14N4, H15N4, H16N4; H1N5, H2N5, H3N5, H4N5, H5N5, H6N5, H7N5, H8N5, H9N5, H10N5, H11N5, H12N5, H13N5, H14N5, H15N5, H16N5; H1N6, H2N6, H3N6, H4N6, H5N6, H6N6, H7N6, H8N6, H9N6, H10N6, H11N6, H12N6, H13N6, H14N6, H15N6, H16N6; H1N7, H2N7, H3N7, H4N7, H5N7, H6N7, H7N7, H8N7, H9N7, H10N7, H11N7, H12N7, H13N7, H14N7, H15N7, H16N7; H1N8, H2N8, H3N8, H4N8, H5N8, H6N8, H7N8, H8N8, H9N8, H10N8, H11N8, H12N8, H13N8, H14N8, H15N8, H16N8; H1N9, H2N9, H3N9, H4N9, H5N9, H6N9, H7N9, H8N9, H9N9, H10N9, H11N9, H12N9, H13N9, H14N9, H15N9, and H16N9). In some embodiments the influenza A subtype is a pandemic influenza A. Exemplary pandemic influenza subtypes include, but are not limited to, H1N1, H2N2, H3N2 and H5N1.

[0018] A list of identified Influenza A strains, including influenza A H1N1 strains, is available from the World Health Organization (WHO) and United States Centers for Disease Control (CDC) databases of Influenza A subtypes. The National Center for Biotechnology Information

(NCBI) database maintained by the United States National Library of Medicine also maintains an updated database describing the length and sequence of HA, M2, NP, M1 and PB1 genes of viruses of influenza A species. Strains listed by these organizations and strains described in other commercial and academic databases, or in literature publications and known in the art, are contemplated for use in the invention. It is also contemplated that additional influenza A strains hereafter identified and isolated are also useful in the invention as sources of influenza A protein sequences. Accordingly, any strain specifically exemplified in the specification and those known or after discovered in the art are amenable to the recombinant vaccinia virus, pharmaceutical compositions, and methods of the invention. Exemplary strains include, but are not limited to, the strains in Table 1 below. The table also lists exemplary genes and associated database accession numbers of those strains.

Table 1

Virus subtype	Inserted Influenza gene	Virus strain	NCBI gene acc no.	NCBI amino acid acc no.
H5N1	HA	A/Viet Nam/1203/2004	AY818135	AAW80717
H5N1	NP	A/Viet Nam/1203/2004	AY818138	AAW80720
H5N1	M1	A/Viet Nam/1203/2004	AY818144	AAW80726
H5N1	PB1	A/Viet Nam/1203/2004	AY818129	AAW80711
H5N1	M2	A/Viet Nam/1203/2004	EF541453	ABP35634
H1N1 sw	M2	A/California/07/09	FJ969537	ACP44185
H1N1	M2	A/New York/3315/2009	CY050765	ACZ05592
H2N2	M2	A/Korea/426/68	NC_007377	YP_308853
H3N2	M2	A/NewYork/392/2004	NC_007367	YP_308840
H9N2	M2	A/chicken/Korea/SH0913/2009	HQ221654	ADQ43641
H7N2	M2	A/New York/107/2003	EU587373	ACC55276
H7N3	M2	A/chicken/Pakistan/34668/1995	CY035834	ACJ03948

[0019] In recombinant viruses of the invention, open reading frames encoding hlHA/M2e, hlHA, NP, M1 and/or PB1 may be codon-optimized for expression in human cells. In these embodiments, one or more (or all) of the naturally occurring codons in an open reading frame have been replaced in the codon-optimized open reading frame with codons frequently used in

genes in human cells (sometimes referred to as preferred codons). Codons may be optimized to avoid repeat sequences to stabilize an open reading frame in the rMVA and/or to avoid unwanted transcription stop signals. Codon-optimization, in general, has been used in the field of recombinant gene expression to enhance expression of polypeptides in cells.

[0020] Gene cassettes encoding hlHA/M2e, hlHA, NP, M1 and PB1 in recombinant viruses of the invention include an open reading frame under the control of (*i.e.*, operatively linked to) a promoter that functions (*i.e.*, directs transcription of the open reading frame) in the recombinant vaccinia viruses. In exemplary embodiments, expression from gene cassettes is under the control of the strong early/late vaccinia virus mH5 promoter (SEQ ID NO: 18) or the synthetic early/late selP promoter (SEQ ID NO: 19) (Chakrabarti *et al.* 1997). In the gene cassettes of the invention the open reading frame is also operatively linked to a transcription stop signal such as a vaccinia virus early transcription stop signal.

[0021] In one aspect, the invention provides recombinant vaccinia virus comprising a gene cassette encoding an influenza A h1HA/M2e fusion protein. In some embodiments, the recombinant vaccinia virus is a recombinant MVA comprising a gene cassette expressing the hlHA/M2e fusion protein set out in SEQ ID NO: 2. In some embodiments, the recombinant vaccinia virus further comprises a gene cassette expressing the M1 protein (for example, the M1 set out in SEQ ID NO: 11) and/or a gene cassette expressing the PB1 protein (for example, the PB1 protein set out in SEQ ID NO: 17).

[0022] In another aspect, the invention provides recombinant vaccinia virus comprising a first gene cassette encoding an influenza A h1HA/M2e fusion protein. and a second gene cassette encoding an influenza NP. In some embodiments, the recombinant vaccinia virus is a recombinant MVA comprising a first gene cassette expressing the hlHA/M2e fusion protein set out in SEQ ID NO: 2 and a second gene cassette expressing the NP set out in SEQ ID NO: 13. In some embodiments, the recombinant vaccinia virus further comprises a gene cassette expressing the M1 protein (for example, the M1 set out in SEQ ID NO: 11) and/or a gene cassette expressing the PB1 protein (for example, the PB1 protein set out in SEQ ID NO: 17).

[0023] In yet another aspect, the invention provides recombinant vaccinia virus comprising a first gene cassette encoding an influenza A h1HA and a second gene cassette encoding an influenza NP. In some embodiments, the recombinant vaccinia virus is a recombinant MVA

comprising a first gene cassette expressing the hlHA set out in SEQ ID NO: 15 and a second gene cassette expressing the NP set out in SEQ ID NO: 13. In some embodiments, the recombinant vaccinia virus further comprises a gene cassette expressing the M1 protein (for example, the M1 set out in SEQ ID NO: 11) and/or a gene cassette expressing the PB1 protein (for example, the PB1 protein set out in SEQ ID NO: 17).

[0024] In recombinant vaccinia viruses of the invention, the gene cassettes may be inserted in non-essential regions of the vaccinia virus genome, such as the deletion I region, the deletion II region, the deletion III region, the deletion IV region, the thymidine kinase locus, the D4R/5R intergenic region, or the HA locus. In exemplified embodiments of recombinant MVA, the insertion of the hlHA/M2e and hlHA gene cassettes is in the D4R/5R intergenic region and the insertion of the NP gene cassette is in the deletion III region. The recombinant MVA is derived from an MVA free of bovine spongiform encephalopathy (BSE) such as MVA74 LVD6 obtained from the National Institutes of Health.

[0025] The recombinant viruses of the invention may be formulated as pharmaceutical compositions according to methods known in the art. In some embodiments, the recombinant viruses are formulated as described in International Publication No. WO 2010/056991.

[0026] The invention provides methods of inducing a heterosubtypic influenza A immune response in an individual comprising administering compositions of recombinant vaccinia virus of the invention to the individual. In the methods, the composition may be administered as a single dose, a double dose or multiple doses. The administration route in humans may be inhalation, intranasally, orally, and parenterally. Examples of parenteral routes of administration include intradermal, intramuscular, intravenous, intraperitoneal and subcutaneous administration. The range of the human immunization dose may be about 10^6 to about 10^9 PFU. The methods of the invention induce humoral and cellular immune responses in the individual. Moreover, in embodiments of the invention the methods induce a protective immune response in the individual. The protective immune response may be where the individual exhibits no symptoms of infection, a reduction in symptoms, a reduction in virus titer in tissues or nasal secretions, and/or complete protection against infection by influenza virus.

[0027] The invention also provides kits for administering recombinant vaccinia virus of the invention packaged in a manner which facilitates their use to practice methods of the invention.

In one embodiment, such a kit includes a recombinant virus or composition described herein, packaged in a container such as a sealed bottle or vessel, with a label affixed to the container or included in the package that describes use of the compound or composition in practicing the method. Preferably, the recombinant virus or composition is packaged in a unit dosage form. The kit may further include a device suitable for administration according to a specific route of administration or for practicing a screening assay. Preferably, the kit contains a label that describes use of the recombinant vaccinia virus. In some embodiments, the kit comprises instructions for administration to a human subject.

[0028] Also provided are methods of producing a recombinant vaccinia virus expressing a gene cassette of the invention. As illustrated with MVA, the methods comprise the steps of: a) infecting primary chicken embryo cells or a suitable permanent cell line (*e.g., avian)* with MVA, b) transfecting the infected cells with a plasmid comprising the gene cassette and comprising DNA flanking the gene cassette that is homologous to a non-essential region of the MVA genome, c) growing the cells to allow the plasmid to recombine with the MVA genome during replication of the MVA in chicken cells thereby inserting the gene cassette into the MVA genome in the non-essential region, and d) obtaining the recombinant MVA produced. Exemplary chicken embryo cells are described in U.S. Patent No. 5,391,491. (Slavik *et al.* 1983) Other avian cells (*e.g.*, DF-1) are also contemplated. In the methods, the non-essential MVA region is the deletion I region, the deletion II region (Meyer *et al.* 1991), the deletion III region (Antoine *et al.* 1996), the deletion IV region (Meyer *et al., supra*; Antoine et al. 1998) the thymidine kinase locus (Mackett *et al.* 1982), the D4R/5R intergenic region (Holzer *et al.* 1998), or the HA locus (Antoine *et al. supra*). In one exemplified embodiment, the insertion is in the deletion III region. In another exemplified embodiment, the insertion is in the D4R/5R intergenic region. If two gene cassettes are to be inserted, the two are inserted in different non-essential regions. Gene cassettes may additionally be inserted into any other suitable genomic region or intergenomic regions.

[0029] Other vertebrate cell lines are useful for culture and growth of vaccinia virus of the invention. Exemplary vertebrate cells useful to culture vaccinia virus of the invention include, but are not limited to, MRC-5, MRC-9, CV-1 (African Green monkey), HEK (human embryonic kidney), PerC6 (human retinoblast), BHK-21 cells (baby hamster kidney), BSC (monkey kidney cell), LLC-MK2 (monkey kidney) and permanent avian cell lines such as DF-1.

[0030] Vero cells are an accepted cell line for production of viral vaccines according to the World Health Organization. In some embodiments, recombinant replicating vaccinia virus of the invention are produced in Vero cells.

[0031] Additional aspects and details of the invention will be apparent from the following examples, which are intended to be illustrative rather than limiting.

Figures

[0032] Figure 1 shows the amino acid sequence (SEQ ID NO: 15) of the headless HA protein encoded by recombinant MVA (rMVA) of the invention. The protein contains a signal sequence (grey), HA1 residues (red), a linker peptide of four glycines (black), HA1 residues (red), and the HA2 stalk region (black). Cysteines 58 and 63 and the polybasic cleavage site (amino acids 112-119) are underlined. Figure 2 shows the nucleotide sequence (SEQ ID NO: 14) of the headless HA protein encoded by rMVA of the invention.

[0033] Figure 3 shows the amino acid sequence (SEQ ID NO: 2) of headless HA/M2e fusion protein. The designed protein contains a signal sequence (grey), HA1 residues (red), a linker peptide of three glycines (black), the M2e of H5N1 (blue), the six amino acid linker GSAGSA (black), the M2e of H1N1 (equivalent to H2N2, H3N2; green), the six amino acid linker GSAGSA, the M2e of H9N2 (orange), the six amino acid linker GSAGSA, the M2e of H7N2 (pink), a linker peptide of three glycines (black), HA1 residues (red) and the HA2 stalk region (black). The polybasic cleavage site (amino acids 224-231) is underlined. Figure 4 shows the nucleotide sequence (SEQ ID NO: 1) of the headless HA/M2e fusion protein encoded by rMVA of the invention.

[0034] Figure 5 shows single-insert rMVAs containing influenza genes. Figure 5A indicates the hlHA, hlHA/M2e, M2, PB1, or M1 gene cassettes that are located in the recombinant MVA D4R/D5R intergenic locus, at the position corresponding to nucleotide 87,281 of wild type MVA (Antoine *et al, supra*). Figure 5B indicates the NP gene cassette is located in the del lll locus at the position corresponding to nucleotide 142,992 of wild type MVA.

[0035] Figure 6 shows a Western Blot of chicken cell lysates tested for influenza virus antigens. A) Expression of headless HA and the headless HA/M2e fusion protein using a detection antibody directed against HA. Lane 1, protein ladder, size in kDa; lane 2, MVA-hlHA;

lane 3, MVA-hlHA/M2e; lane 4, MVA wt (negative control); and lane 5, MVA-HA-VN (positive control). B) Expression of the headless HA/M2e fusion protein using a detection antibody directed against M2. Lane 1, protein ladder, size in kDa; lane 2, MVA-M2-VN; lane 3, MVA-hlHA/M2e; and lane 4, MVA wt (negative control). The recombinant MVA-M2-VN expresses the M2 protein (weak band below 15 kDa). The anti-M2-antibody binds a peptide at the N-terminus of the M2 protein; thus the expression of the hlHA/M2e fusion protein is also detectable at around 70 kDa (lane 3).

[0036] Figure 7 shows double-insert rMVAs containing influenza genes. The hlHA or hlHA/M2e gene cassette is located in the D4R/D5R intergenic locus, at the position corresponding to nucleotide 87,281 of wild type MVA. The NP gene cassette is located in the del lll locus at the position corresponding to nucleotide 142,992 of the wild type MVA.

[0037] Figure 8 shows a Western Blot of chicken cell lysates tested for influenza virus antigens. A) Expression of headless HA and the headless HA/M2e fusion protein using a detection antibody directed against HA. Lanes 1 and 7, protein ladder, size in kDa; lane 2, MVA-HA-VN (positive control); lane 3, MVA-hlHA; lane 4, MVA wt (negative control); lane 5, MVA-hlHA/M2e-NP; and lane 6, MVA-hlHA-NP. The hlHA/M2e fusion protein expressed by MVA-hlHA/M2e is visible at around 70 kDa (lane 5). The lower bands at around 40 kDa represent the hlHA expressed by MVA-hlHA-NP and MVA-hlHA. The control construct (MVA-HA-VN), expressing the full length HA protein express the HA0 (band around 80 kDa), the HA1 (band around 55 kDa, and the HA2 (band around 25 kDa). The expression of the HA2 protein is also visible in lanes 3, 5 and 6 as the hlHA and hlHA/M2e proteins also contain the polybasic cleavage site. The specific HA bands are absent in the negative control (lane 4). **B)** NP expression detected with an NP-specific antibody. Lane 1, protein ladder, size in kDa; lane 2, MVA-D3-NP-VN; lane 3, MVA-hlHA-NP; lane 4, MVA-hlHA/M2e-NP; and lane 5, MVA wt (negative control).

[0038] Figure 9 shows monitoring of weight (A, B), clinical symptoms (C, D) and survival (E, F) after vaccination with recombinant MVAs and challenge with H5N1. As controls, mice were vaccinated with MVA-HA-VN, expressing the full-length HA of A/Vietnam/1203/2004, wt MVA or were treated with PBS (panels A, C, E). Mice were vaccinated with the single recombinant MVA-hlHA, MVA-hlHA/M2e, MVA-NP-VN or the double recombinants MVA-

hlHA-NP and MVA-hlHA/M2e-NP (panels B, D, F). After challenge with wild-type H5N1, mice were monitored for 14 days.

[0039] Figure 10 shows monitoring of weight (A, B), clinical symptoms (C, D) and survival (E, F) after vaccination with recombinant MVAs and challenge with H9N2 virus. As controls, mice were vaccinated with the whole virus preparation of H9N2, wt MVA or were treated with PBS (panels A, C, E). Mice were vaccinated with the single recombinant MVA-hlHA, MVA-hlHA/M2e, MVA-NP-VN or double recombinant MVA-hlHA-NP and MVA-hlHA/M2e-NP (panels B, D, F). After challenge with virulent mouse-adapted H9N2 influenza virus, mice were monitored for 14 days.

[0040] Figure 11 shows triple-insert rMVAs containing influenza genes. The hlHA or hlHA/M2e and M1gene cassettes will be located in the D4R/D5R intergenic locus, at the position 87,281 nt of the wt MVA sequence. The NP gene cassette will be located in the del lll locus at the position 142,992 nt of the wt MVA sequence.

Examples

[0041] The present invention is illustrated by the following examples wherein Example 1 describes the choice and design of influenza A antigens in exemplary recombinant MVA of the invention, Example 2 details the production of single-insert recombinant MVAs, Example 3 describes animal experiments with the single-insert MVAs, Example 4 details the production of double-insert recombinant MVAs, Example 5 describes animal experiments with the double-insert MVAs, Example 6 details the production of triple-insert recombinant MVAs and Example 7 describes animal experiments with the triple-insert MVAs.

Example 1

Choice and design of influenza A antigens

[0042] Influenza headless HA, a headless HA/M2e fusion protein, NP, M1, M2 and PB1 were the influenza A antigens chosen to be encoded by exemplary recombinant MVA of the invention.

[0043] Monoclonal antibodies against the HA stalk domain, the HA2 region, are broadly cross-reactive and neutralize several subtypes of viruses (Ekiert *et al.* 2009; Kashyap *et al.* 2008; Okuno *et al.* 1993; Sanchez-Fauquier *et al.* 1987; Sui *et al.* 2009; Throsby *et al.* 2008). The

antibodies target the HA2 region of the molecule and presumably act by preventing the conformational change of HA at low pH, thus presumably blocking fusion of viral and host membranes during influenza infection. However, the production of soluble, native (neutral pH-like) HA2 immunogen has proven to be difficult, owing to the metastable nature of HA (Chen *et al.* 1995). To induce an immune response against the neutral pH conformation, a headless HA was chosen as an antigen. The headless HA consists of two HA1 regions that interact with an HA2 subunit, stabilizing the neutral pH conformation (Bommakanti *et al., supra*; Steel *et al., supra*).

[0044] The extracellular domain of the M2 protein (M2e, 23AS) is highly conserved across influenza A virus subtypes. In animals, M2e specific antibodies reduce the severity of infection with a wide range of influenza A virus strains (Fan *et al.*2004; Neirynck *et al.* 1999). Many groups have reported M2e-based vaccine candidates in different forms (De Filette *et al.* 2008; Denis *et al.* 2008; Eliasson *et al.* 2008; Fan *et al., supra*; Neirynck *et al., supra*). Recently, Zhao *et al.* reported that a tetra-branched multiple antigenic peptide vaccine based on H5N1 M2e induced strong immune responses and cross protection against different H5N1 clades and even heterosubtypic protection from 2009 H1N1 (Zhao *et al.* 2010b; Zhao *et al.* 2010a).

[0045] Vaccination using vectors expressing the conserved influenza NP, or a combination of NP and matrix protein has been studied in animal models and various degrees of protection against both homologous and heterologous viruses have been demonstrated (Price *et al., supra*; Ulmer *et al.* 1993). NP elicit a robust $CD8^+$ T cell response in mice and in humans (McMichael *et al.*, 1986; Yewdell *et al.*, 1985) that, as epidemiological studies suggest, may contribute to resistance against severe disease following influenza A virus infection (Epstein 2006).

[0046] The headless HA included in rMVA of the invention is a new headless HA (hlHA) based on the VN/1203 influenza strain. The hlHA contains a polybasic cleavage site which is cleaved during expression from the rMVA exposing the fusion peptide for the immune system. The amino acid sequence of the hlHA is set out in Figure 1 and in SEQ ID NO: 15. The nucleotide sequence of the MVA insert is set out Figure 2 and SEQ ID NO: 14.

[0047] The amino acid sequence of the headless HA/M2e fusion protein included in rMVA of the invention is set out in Figure 3 below and in SEQ ID NO: 2. The nucleotide sequence of the fusion protein is set out in Figure 4 below and in SEQ ID NO: 1. In the fusion protein, the M2e

domains of H5N1, H9N2, H7N2 and H1N1 (equivalent to H2N2, H3N2) form an M2e "head" on the hlHA. The four particular M2e domains were chosen to represent the M2e from seasonal and pandemic strains.

Example 2

Construction and characterization of single-insert MVA vectors

[0048] The following single-insert, recombinant MVA (rMVA) are utilized in the experiments described herein.

Table 2

rMVA	Inserted influenza gene	NCBI gene acc no.
1. MVA-hlHA	headless HA	based on AY818135
2. MVA-hlHA/M2e	headless HA/M2e fusion	based on AY818135
3. MVA-M1-VN	Matrix protein 1	AY818144
4. MVA-M2-VN	Matrix protein 2	EF541453
5. MVA-PB1-VN	Polymerase subunit PB1	AY818129
6. MVA-mNP	Nucleoprotein	AY818138
7. Control MVA-HA-VN	Hemagglutinin	AY818135
8. Control MVA-wt	No insert	-
9. Control PBS	No insert	-

[0049] For construction of single-insert rMVA vectors expressing hlHA, the hlHA/M2e fusion protein or PB1, the hlHA, hlHA/M2e and PB1 genes were chemically synthesized (Geneart, Inc., Regensburg, Germany). The synthetic genes are driven by the strong vaccinia early/late promoter mH5 (Wyatt *et al.* 1996) and terminated with a vaccinia virus specific stop signal downstream of the coding region that is absent internally. The gene cassettes were cloned in the plasmid pDM-D4R (Ricci et al., 2011) resulting in plasmids pDM-hlHA, pDM-hlHA/M2e and pDM-PB1-VN, respectively. The introduction of the foreign genes into the D4R/D5R intergenic region of MVA was done as described elsewhere (Ricci *et al.* 2011) resulting in viruses MVA-hlHA, MVA-hlHA/M2e, MVA-PB1-VN.

[0050] For the construction of the rMVA expressing M1, the M1 sequence (accession number AY818144) was placed downstream of the strong vaccinia early/late promoter selP (Chakrabarti *et al.* 1997) and cloned in pDM-D4R, resulting in pDM-M1-VN. The expression cassette of

pDD4-M2-VN - including the M2 sequence (accession number EF541453) under the control of the mH5 promoter – was cloned in pDM-D4R resulting in pDM-M2-VN. The plasmids were then used for recombination with MVA according to Holzer *et al, supra* resulting in the viruses MVA-M1-VN and MVA-M2-VN, respectively as shown in Figure 5A.

[0051] For the construction of single-insert MVAs expressing the NP protein, the NP expression cassette of pDD4-mH5-mNP-VN (Mayrhofer *et al., supra*) was cloned in plasmid pd3-lacZ-gpt, resulting in pd3-lacZ-mH5-NP-VN. Plasmid pd3-lacZ-gpt contains a lacZ/gpt selection marker cassette and a multiple cloning site (MCS) for insertion of genes of interest. The sequences are framed by genomic MVA sequences of the del III region. The marker cassette is destabilized by a tandem repeat of MVA del III flank, thus the final recombinant is free of any auxiliary sequences. The insertion plasmid directs the gene cassettes into the MVA deletion III (del III) region. After infection of primary chicken embryo cells with MOI 1, cells were transfected with pd3-lacZ-mH5-NP-VN according to the calcium phosphate technique (Graham and van der Eb 1973), resulting MVA-NP-VN shown in Figure 5B. The MVA strain (MVA 1974/NIH clone 1) was kindly provided by B. Moss (National Institutes of Health).Recombinant virus is selected using the transient marker stabilization method (Scheiflinger *et al, 1998*).

[0052] The single-insert MVA vectors expressing the NP, PB1, M1, M2, hlHA, and hlHA/M2e were characterized by PCR and Western blot as described in Hessel *et al, supra.* Recombinant viruses were grown in CEC or DF-1 cells and purified by centrifugation through a sucrose cushion. Primary CEC were produced in-house and cultivated in Med199 (Gibco®) supplemented with 5 % fetal calf serum (FCS). The DF-1 (CRL-12203) cell line was obtained from the ATCC (American Type Culture Collection) and cultivated in DMEM (Biochrom, Inc.) supplemented with 5 % FCS.

[0053] The correct expression of the influenza proteins by the rMVAs was confirmed by Western blotting. For this purpose CEC or the permanent chicken cell line DF-1 were infected with a MOI of 0.1 and cell lysates were prepared 48-72 hrs post infections. The recombinant MVAs that express the hlHA (MVA-hlHA and MVA-hlHA/M2e) were analyzed in a Western blot using an anti-influenza A/Vietnam/1194/04 (H5N1) polyclonal serum (NIBSC 04/214) for detection. Donkey-anti-sheep alkaline phosphatase-conjugated IgG (Sigma Inc.) was used as a

secondary antibody. The recombinant MVAs that express the M2 and M2e (MVA-M2-VN and MVA-hlHA/M2e) were analyzed in Western Blots using an anti-avian influenza M2 antibody binding a peptide present at the amino terminus of the H5N1 M2 (ProSci, Cat# 4333). Goat-anti-rabbit alkaline phosphatase-conjugated IgG (Sigma Inc.) antibody was used as a secondary antibody. As shown in Figure 6A, the recombinant MVAs expressing the hlHA (MVA-hlHA and MVA-hlHA/M2e) gene inserts induced expression of the HA containing antigens in avian DF-1 cells. The bands around 40 kDa in lane 2 represent the hlHA. The larger band at around 70 kDa in lane 3 represents the hlHA/M2e. The large band at around 80 kDa in lane 5 represents the HA0 hemagglutinin-precursor, which is cleaved into the HA1 and HA2 subunits represent by the bands at approximately 55 and 25 kDa. The specific hlHA, hlHA/M2e or HA bands are absent in the wild-type MVA control (lane 4).

[0054] Figure 6B shows the M2 expression by MVA-M2-VN (lane 2) or MVA-hlHA/M2e (lane 3). The weak but specific band around 10 kDa in lane 2 represents the wild-type M2 protein whereas the larger band around 70 kDa represents the hlHA/M2e protein. Both bands are absent in the wild-type MVA control (lane 4).

[0055] The expression of the M1, NP and PB1 protein is detected with polyclonal guinea-pig anti-influenza H5N1 serum produced in house, a polyclonal goat antibody detecting the PB1 of Influenza A virus (Santa Cruz, Cat#: vC-19), and a monoclonal mouse-anti-NP-antibody (BioXcell, Cat# BE0159), respectively. The MVA-M1-VN and MVA-NP-VN induce expression of the M1 protein (around 27 kDa) and the NP protein (around 60 kDa) (not shown).

Example 3

Animal experiments with the single-insert vaccines

Protection experiment

[0056] A standard protection experiment consists of two arms (primed with about $1x10^3$-$1x10^5$ $TCID_{50}$ H1N1v CA/07 and unprimed) of nine groups of mice each (respectively vaccinated i.m. with $1x10^6$ pfu of the nine vaccines and controls shown in Table 2), a group consisting of six animals resulting in 108 animals, defines one set. The animals of one set are challenged with one of the six challenge viruses shown in Table 3 below.

Table 3

Pre-treatment	Challenge strain	Subtype	Abbreviation
H1N1v /unprimed	A/California/07/2009	H1N1	CA/07
H1N1v /unprimed	A/Vietnam/1203/2004	H5N1	VN/1203
H1N1v /unprimed	A/HongKong/G9/	H9N2	HK/G9
H1N1v /unprimed	A/Victoria/210/2009	H3N2	VI/09
H1N1v /unprimed	A/FPV/Rostock/34	H7N1	RO/34
H1N1v /unprimed	A/PR8/1934	H1N1	PR8

[0057] Female Balb/c mice are 8-10 weeks old at the pre-treatment time point and 14-16 weeks old at the time point of immunization with the vaccines and controls shown in Table 2. Mice were immunized intramuscularly twice (days 42 and 63) with 10^6 pfu of the vaccines or wild type MVA, 3.75 µg whole virus preparation H9N2 A/HongKong/G9/1997 or with buffer (PBS). At day 84, mice were challenged intranasally with 10^3 $TCID_{50}$ H5N1 A/Vietnam/1203/2004 (H5N1, CDC #2004706280), with $2.5x10^4$ $TCID_{50}$ mouse adapted H9N2 A/HongKong/G9/1997 or with $1.66x10^4$ $TCID_{50}$ H7N1 A/FPV/Rostock/34. The challenge doses correspond to approx. 30 LD50 for the H5N1 challenge and 32 LD50 for the H9N2 challenge per animal. Sera are collected at days 41, 62 and 85 and analyzed for HA-specific IgG concentration by HI titer or microneutralization assay.

[0058] The primary outcome of the animal experiments is protection as measured by lethal endpoint, weight loss, or lung titer. Further the ELISA titers of pooled pre-challenge sera measured against inactivated whole virus H5N1 strain A/Vietnam/1203/2004 are determined.

T cell experiments

[0059] Frequencies of influenza-specific CD4 and CD8 T cells are determined in immunized mice by flow cytometry. In a standard experiment, groups of 5 female BALB/c mice are immunized twice with the vaccines or controls listed in Table 2. Splenocytes are re-stimulated in-vitro using inactivated whole virus antigens of different influenza strains for CD4 T-cells and, when available, peptides representing the CD8 T-cell epitopes of the vaccine insert constructs and IFN-γ production are measured. All experiments are performed twice, using a total of 140 animals.

Other experiments

[0060] An evaluation of the cell-mediated immunity after a single immunization, demonstration of functional activity of cytotoxic T-cells in a VITAL assay and assessment of recruitment of influenza-specific T-cells into the lungs of challenged animals are also carried out. The induction / expansion of vaccine-specific T-cells is also monitored in the primed mouse model by immunizing mice which resolved a influenza virus infection once with these vaccines.

.

Example 4

Construction and characterization of double-insert rMVA vectors

[0061] The following double-insert, rMVA and controls are utilized in the experiments described herein.

Table 4

rMVA	Inserted influenza gene(s)	Comment
1. MVA-hlHA-NP	headless HA + NP	Double insert construct
2. MVA-hlHA/M2e-NP	headless HA/m2e fusion protein + NP	Double insert construct
3. MVA-NP-VN	nucleoprotein	Control
4. MVA-HA-VN	hemagglutinin	Control
5. MVA-wt	empty vector	Neg. control
6. PBS	-	Neg. control

[0062] For the construction of the double insert rMVA vector co-expressing either the hlHA or hlHA/M2e gene cassette in combination with the NP protein gene cassette, the single insert MVA recombinants of Example 2 containing the hlHA or hlHA/M2e gene cassette are used. CEC cells were infected with MVA-hlHA or MVA-hlHA/Me2 and afterwards transfected with pd3-lacZ-mH5-NP-VN (see Example 2). Homologous recombination and propagation of the recombinant MVA vectors are performed as described in Example 2. The resulting double insert MVA vectors, named MVA-hlHA-NP or MVA-hlHA/M2e-NP, contain the hlHA or hlHA/M2e expression cassette in the D4R/D5R locus and the NP expression cassette in the del III locus. See Figure 7.

[0063] The recombinant MVAs were characterized by Western Blot as described in Example 2. Figure 8A shows the expression of the hlHA and hlHA/M2e after infection of CEC with MVA-hlHA-NP (lane 6) or MVA-hlHA/M2e-NP (lane 5). The bands around 40 kDa in lanes 3 and 6 represent the hlHA of the MVA-hlHA and MVA-hlHA-NP constructs. The band around 70 kDa in lane 5 represents the hlHA/M2e fusion protein. The HA bands are absent in the wild-type control in lane 4. The same samples were used for detection of NP protein expression in Western Blots (as described in Example 2). As shown in Figure 8B, the recombinant MVAs MVA-hlHA-NP and MVA-hlHA/M2e-NP also induced expression of the NP protein in avian CEC cells. The bands around 60 kDa in lanes 2 to 4 represent the NP.

Example 5

Animal experiments with the double-insert vaccines or vector combinations

Protection experiment

[0064] A standard experiment included eight groups of mice (vaccinated with the six vaccines and controls shown in Table 5) each group consisting of six animals. The protection experiments were carried out as described in Example 3. After challenge mice were monitored over a time period of 14 days and weight loss or symptoms including ruffled fur (score of 1), curved posture (score of 2), apathy (score of 3), and death (score of 4) were recorded. For ethical reasons, mice were euthanized after weight loss of ≥25%. Protection results are compiled in Table 5 and displayed in Figures 9 and 10.

Table 5. *Protection of mice from death after double dose vaccinations with recombinant MVAs and homologous or heterologous challenge.*

Gr.	Vaccine	After H5N1 VN1203[(1)] challenge		After H9N2 HK/G9[(2)] challenge	
		Clinical score at day 14	Protection n/nt[(3)] (%)	Clinical score at day 14	Protection n/nt (%)
1	MVA-hlHA-NP	2.83	2/6 (33)	0	6/6 (100)
2	MVA-hlHA/M2e-NP	1	5/6 (83)	0	6/6 (100)
3	MVA-hlHA	2.67	2/6 (33)	3.33	1/6 (17)
4	MVA-hlHA/M2e	4	0/6 (0)	2.67	2/6 (33)
5	MVA-NP-VN	3.33	2/6 (33)	0	6/6 (100)
6	Homologous control vaccine[(4)]	0	6/6 (100)	0	6/6 (100)
7	MVA-wt[(5)]	4	0/6 (0)	2.83	2/6 (33)
8	PBS	2.67	2/6 (33)	4	0/6 (0)

[(1)]VN1203, challenge strain A/Vietnam/1203/2004; [(2)]HK/G9, challenge strain A/HongKong/G9/1997; [(3)] n/nt, survival per group, [(4)]Homologous control vaccine; [(5)]wild-type MVA (NIH74 LVD clone 6).

[0065] As positive control mice were vaccinated with homologous control constructs. In case of H5N1 challenge mice were vaccinated with MVA-HA-VN (Hessel *et al.*, 2011) and in case of H9N2 challenge mice were vaccinated with an inactivated whole virus preparation of the H9N2 A/HongKong/G9/1997 influenza virus. Both controls induced full protection (Table 5; Figure 9 and 10, panels E). In the wild-type MVA and buffer groups all mice showed marked weight loss compared to the positive control groups and nearly all mice died after challenge. Mice

vaccinated with the single recombinant MVAs (MVA-hlHA, MVA-hlHA/M2e, MVA-D3-NP-VN) showed no significantly better protection after the strong H5N1 challenge compared to the negative control groups (Figure 9 A-F). Also against heterosubtypic (H9N2) challenge no significant protection was seen in MVA-hlHA and MVA-hlHA/M2e vaccinated groups (Fig. 10).

[0066] Surprisingly, however, vaccination with the double construct expressing the fusion protein hlHA/M2e and the NP protein resulted in nearly full protection (Figure 9 B, D, F) after the H5N1 challenge with approx. 30 LD50 per animal. Also after heterosubtypic challenge (with approx. 32 LD50 H9N2 virus) mice were fully protected after vaccination with the double recombinant MVA-hlHA/M2e-NP. Furthermore, the double recombinant MVA-hlHA-NP and the single recombinant MVA-NP-VN induced full protection against the heterosubtypic challenge with H9N2 (Fig. 10, B, D, F). As can be seen in the weight monitoring (Figs. 9 and 10, panels B) and in the clinical scores (Figs. 9 and 10, panels D), the double construct MVA-hlHA/M2e-NP showed the best results presumably by combined beneficial effects contributed by the different influenza antigens.

T cell experiments

[0067] Frequencies of influenza-specific CD4 and CD8 T cells are determined in immunized mice by flow cytometry. In a standard protocol experiment, groups of 5 female BALB/c mice are immunized twice with the vaccines or controls listed in Table 4. Splenocytes are re-stimulated in-vitro using inactivated whole virus antigens of different influenza strains for CD4 T-cells and, when available, peptides representing the CD8 T-cell epitopes of the vaccine insert constructs and IFN-γ production are measured. All experiments are performed twice.

Other experiments

[0068] An evaluation of the cell mediated immunity after a single immunization, demonstration of functional activity of cytotoxic T-cells in a VITAL assay and assessment of recruitment of influenza-specific T-cells into the lungs of challenged animals are also carried out. The induction / expansion of vaccine-specific T-cells is also monitored in the primed mouse model by immunizing mice which resolved a influenza virus infection once with these vaccines.

Example 6

Construction and characterization of triple-insert rMVA vectors and virus-like particles

[0069] Influenza virus-like particles (VLPs) induce humoral and cellular responses and can protect against lethal challenges (Bright *et al.* 2007; Pushko *et al.* 2005; Song *et al.* 2010). VLPs chosen for experiments herein comprise either hlHA or hlHA/M2e in combination with NP and M1. The VLPs are generated from triple-insert MVA vectors.

[0070] For the construction of the triple-insert MVA vectors co-expressing either hlHA or hlHA/M2e in combination with the M1 (SEQ ID NO: 11) and the NP protein (SEQ ID NO: 13), the M1 gene (SEQ ID NO: 10) of pDD4-M1-VN is cloned downstream of the synthetic early/late promotor selP (Chakrabarti *et al.* 1997). The resulting gene cassette is cloned downstream of the hlHA or hlHA/M2e gene cassette in pDM-hlHA or pDM-hlHA/M2e. The resulting plasmids harboring a double gene cassette (pDM-hlHA-M1 and pDM-hlHA/M2e-M1) are used for recombination into defective MVA as described above. Afterwards, a recombination with an NP gene cassette (SEQ ID NO: 12)-containing plasmid (pD3-lacZ-gpt-NP-VN) is done resulting in a triple-insert MVA virus. This triple-insert MVA is plaque purified under transient marker selection.

[0071] The triple-insert MVA vectors, named MVA-hlHA-M1-NP or MVA-hlHA/M2e-M1-NP contain the hlHA or hlHA/M2e expression cassette and M1 expression cassette in tandem order in the D4R/D5R locus and the NP expression cassette in the del III locus (Figure 7).

[0072] Detection of VLPs is as follows. HeLa or 293 cells are seeded into T175cm^2 flasks and grown in DMEM + 10%FCS + Pen/Strep. To generate VLPs, cells are infected with 1 MOI of single-insert MVA or triple-insert MVA recombinants, respectively. Empty MVA vectors or single-insert MVA recombinants without M1 are used as controls. At 1h post infection (p.i.), the medium is replaced by DMEM + Pen/Strep and culture medium is harvested 48h p.i. and cellular debris is pelleted by centrifugation at 2.000 x g for 10 min. The procedure for analyzing VLPs by sucrose gradient density flotation and sucrose cushion has been described previously (Chen *et al.* 2007; Chen *et al.* 2005; Gomez-Puertes *et al.* 2000). The samples are then analyzed by immunoblotting. Additionally, electron microscopy (EM) analysis with medium of infected cells is performed.

Example 7

Animal experiments with the triple-insert vaccines or vector combinations

[0073] A standard experiment includes 6 groups of primed and unprimed mice (vaccinated with the 6 vaccines and controls shown in Table 5), each group consisting of 6 animals, resulting in 36 animals (1 set). The animals are challenged with one of the 6 challenge viruses shown in Table 3. In sum, there are 6 sets of 72 animals each requiring 432 mice to assess cross-protection in the primed and naive models.

Table 5

rMVA	**Inserted influenza gene(s)**	**comment**
1. MVA-hlHA-M1-NP	headless HA + nucleoprotein + matrix 1	3 inserts
2. MVA-hlHA/M2e-M1-NP	headless HA/m2e fusion protein + nucleoprotein + matrix 1	3 inserts
3. MVA-tbd	best construct from previous screening	control
4. MVA-HA-VN	hemagglutinin	control
5. MVA-wt	empty vector	neg. control
6. PBS	-	neg. control

[0074] The present invention is illustrated by the foregoing examples and variations thereof will be apparent to those skilled in the art. Therefore, no limitations other than those set out in the following claims should be placed on the invention.

[0075] All documents cited in this application are hereby incorporated by reference in their entirety for their disclosure described.

LITERATURE CITATIONS

Antoine, G., F. Scheiflinger, F. Dorner, and F. G. Falkner. 1998. The complete genomic sequence of the modified vaccinia Ankara strain: comparison with other orthopoxviruses. Virology 244:365-396.

Antoine,G., Scheiflinger,F., Holzer,G., Langmann,T., Falkner,F.G., and Dorner,F. 1996. Characterization of the vaccinia MVA hemagglutinin gene locus and its evaluation as an insertion site for foreign genes. Gene 177:43-46.

Assarsson, E., H. H. Bui, J. Sidney, Q. Zhang, J. Glenn, C. Oseroff, I. N. Mbawuike, J. Alexander, M. J. Newman, H. Grey, and A. Sette. 2008. Immunomic analysis of the repertoire of T-cell specificities for influenza A virus in humans. J Virol 82:12241-51.

Berthoud, T. K., M. Hamill, P. J. Lillie, L. Hwenda, K. A. Collins, K. J. Ewer, A. Milicic, H. C. Poyntz, T. Lambe, H. A. Fletcher, A. V. Hill, and S. C. Gilbert. 2011. Potent CD8+ T-cell immunogenicity in humans of a novel heterosubtypic influenza A vaccine, MVA-NP+M1. Clin Infect Dis 52:1-7.

Bommakanti, G., M. P. Citron, R. W. Hepler, C. Callahan, G. J. Heidecker, T. A. Najar, X. Lu, J. G. Joyce, J. W. Shiver, D. R. Casimiro, J. ter Meulen, X. Liang, and R. Varadarajan. 2010. Design of an HA2-based Escherichia coli expressed influenza immunogen that protects mice from pathogenic challenge. Proc Natl Acad Sci U S A 107:13701-6.

Bright, R. A., D. M. Carter, S. Daniluk, F. R. Toapanta, A. Ahmad, V. Gavrilov, M. Massare, P. Pushko, N. Mytle, T. Rowe, G. Smith, and T. M. Ross. 2007. Influenza virus-like particles elicit broader immune responses than whole virion inactivated influenza virus or recombinant hemagglutinin. Vaccine 25:3871-8.

Brookes,R.H., Hill,P.C., Owiafe,P.K., Ibanga,H.B., Jeffries,D.J., Donkor,S.A., Fletcher,H.A., Hammond,A.S., Lienhardt,C., Adegbola,R.A., McShane,H., and Hill,A.V. 2008. Safety and immunogenicity of the candidate tuberculosis vaccine MVA85A in West Africa. PLoS One. 3:e2921.

Carroll, M. W., and B. Moss. 1997. Poxviruses as expression vectors. Curr Opin Biotechnol 8:573-7.

Cebere,I., Dorrell,L., McShane,H., Simmons,A., McCormack,S., Schmidt,C., Smith,C., Brooks,M., Roberts,J.E., Darwin,S.C., Fast,P.E., Conlon,C., Rowland-Jones,S., McMichael,A.J., and Hanke,T. 2006. Phase I clinical trial safety of DNA- and modified virus Ankara-vectored human immunodeficiency virus type 1 (HIV-1) vaccines administered alone and in a prime-boost regime to healthy HIV-1-uninfected volunteers. Vaccine 24:417-425.

Chakrabarti, S., J. R. Sisler, and B. Moss. 1997. Compact, synthetic, vaccinia virus early/late promoter for protein expression. Biotechniques 23:1094-7.

Chen, B. J., G. P. Leser, E. Morita, and R. A. Lamb. 2007. Influenza virus hemagglutinin and neuraminidase, but not the matrix protein, are required for assembly and budding of plasmid-derived virus-like particles. J.Virol. 81:7111-7123.

Chen, B. J., M. Takeda, and R. A. Lamb. 2005. Influenza virus hemagglutinin (H3 subtype) requires palmitoylation of its cytoplasmic tail for assembly: M1 proteins of two subtypes differ in their ability to support assembly. J Virol 79:13673-84.

Chen, J., S. A. Wharton, W. Weissenhorn, L. J. Calder, F. M. Hughson, J. J. Skehel, and D. C. Wiley. 1995. A soluble domain of the membrane-anchoring chain of influenza virus hemagglutinin (HA2) folds in Escherichia coli into the low-pH-induced conformation. Proc Natl Acad Sci U S A 92:12205-9.

De Filette, M., W. Martens, K. Roose, T. Deroo, F. Vervalle, M. Bentahir, J. Vandekerckhove, W. Fiers, and X. Saelens. 2008. An influenza A vaccine based on tetrameric ectodomain of matrix protein 2. J Biol Chem 283:11382-7.

Denis, J., E. Acosta-Ramirez, Y. Zhao, M. E. Hamelin, I. Koukavica, M. Baz, Y. Abed, C. Savard, C. Pare, C. Lopez Macias, G. Boivin, and D. Leclerc. 2008. Development of a universal influenza A vaccine based on the M2e peptide fused to the papaya mosaic virus (PapMV) vaccine platform. Vaccine 26:3395-403.

Ekiert, D. C., G. Bhabha, M. A. Elsliger, R. H. Friesen, M. Jongeneelen, M. Throsby, J. Goudsmit, and I. A. Wilson. 2009. Antibody recognition of a highly conserved influenza virus epitope. Science 324:246-51.

Eliasson, D. G., K. El Bakkouri, K. Schon, A. Ramne, E. Festjens, B. Lowenadler, W. Fiers, X. Saelens, and N. Lycke. 2008. CTA1-M2e-DD: a novel mucosal adjuvant targeted influenza vaccine. Vaccine 26:1243-52.

Epstein, S. L. 2006. Prior H1N1 influenza infection and susceptibility of Cleveland Family Study participants during the H2N2 pandemic of 1957: an experiment of nature. J Infect Dis 193:49-53.

Fan, J., X. Liang, M. S. Horton, H. C. Perry, M. P. Citron, G. J. Heidecker, T. M. Fu, J. Joyce, C. T. Przysiecki, P. M. Keller, V. M. Garsky, R. Ionescu, Y. Rippeon, L. Shi, M. A. Chastain, J. H. Condra, M. E. Davies, J. Liao, E. A. Emini, and J. W. Shiver. 2004. Preclinical study of influenza virus A M2 peptide conjugate vaccines in mice, ferrets, and rhesus monkeys. Vaccine 22:2993-3003.

Gomez-Puertas, P., C. Albo, E. Perez-Pastrana, A. Vivo, and A. Portela. 2000. Influenza virus matrix protein is the major driving force in virus budding. J Virol 74:11538-47.

Graham, F. L., and A. J. van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52:456-67.

Greenbaum, J. A., M. F. Kotturi, Y. Kim, C. Oseroff, K. Vaughan, N. Salimi, R. Vita, J. Ponomarenko, R. H. Scheuermann, A. Sette, and B. Peters. 2009. Pre-existing immunity against swine-origin H1N1 influenza viruses in the general human population. Proc Natl Acad Sci U S A 106:20365-70.

Hessel, A., M. Schwendinger, D. Fritz, S. Coulibaly, G. W. Holzer, N. Sabarth, O. Kistner, W. Wodal, A. Kerschbaum, H. Savidis-Dacho, B. A. Crowe, T. R. Kreil, P. N. Barrett, and F. G. Falkner. 2010. A pandemic influenza H1N1 live vaccine based on modified vaccinia Ankara is highly immunogenic and protects mice in active and passive immunizations. PLoS One 5:e12217.

Hessel et al., 2011. PLoS ONE 6(1): e16247. doi:10.1371/journal.pone.0016247

Hoelscher, M. A., S. Garg, D. S. Bangari, J. A. Belser, X. Lu, I. Stephenson, R. A. Bright, J. M. Katz, S. K. Mittal, and S. Sambhara. 2006. Development of adenoviral-vector-based pandemic influenza vaccine against antigenically distinct human H5N1 strains in mice. Lancet 367:475-481.

Hoelscher, M. A., L. Jayashankar, S. Garg, V. Veguilla, X. Lu, N. Singh, J. M. Katz, S. K. Mittal, and S. Sambhara. 2007. New pre-pandemic influenza vaccines: an egg- and adjuvant-independent human adenoviral vector strategy induces long-lasting protective immune responses in mice. Clin. Pharmacol. Ther. 82:665-671.

Holzer, G. W., W. Gritschenberger, J. A. Mayrhofer, V. Wieser, F. Dorner, and F. G. Falkner. 1998. Dominant host range selection of vaccinia recombinants by rescue of an essential gene. Virology 249:160-6.

Kashyap, A. K., J. Steel, A. F. Oner, M. A. Dillon, R. E. Swale, K. M. Wall, K. J. Perry, A. Faynboym, M. Ilhan, M. Horowitz, L. Horowitz, P. Palese, R. R. Bhatt, and R. A. Lerner. 2008. Combinatorial antibody libraries from survivors of the Turkish H5N1 avian influenza outbreak reveal virus neutralization strategies. Proc Natl Acad Sci U S A 105:5986-91.

Kreijtz, J. H., M. G. de, C. A. van Baalen, R. A. Fouchier, A. D. Osterhaus, and G. F. Rimmelzwaan. 2008. Cross-recognition of avian H5N1 influenza virus by human cytotoxic T-lymphocyte populations directed to human influenza A virus. J.Virol. 82:5161-5166.

Kreijtz, J. H., Y. Suezer, G. de Mutsert, G. van Amerongen, A. Schwantes, J. M. van den Brand, R. A. Fouchier, J. Lower, A. D. Osterhaus, G. Sutter, and G. F. Rimmelzwaan. 2009. MVA-based H5N1 vaccine affords cross-clade protection in mice against influenza A/H5N1 viruses at low doses and after single immunization. PLoS One 4:e7790.

Kreijtz, J. H., Y. Suezer, G. de Mutsert, J. M. van den Brand, G. van Amerongen, B. S. Schnierle, T. Kuiken, R. A. Fouchier, J. Lower, A. D. Osterhaus, G. Sutter, and G. F. Rimmelzwaan. 2009. Preclinical evaluation of a modified vaccinia virus Ankara (MVA)-based vaccine against influenza A/H5N1 viruses. Vaccine 27:6296-9.

Kreijtz, J. H., Y. Suezer, G. de Mutsert, J. M. van den Brand, G. van Amerongen, B. S. Schnierle, T. Kuiken, R. A. Fouchier, J. Lower, A. D. Osterhaus, G. Sutter, and G. F. Rimmelzwaan. 2009. Recombinant modified vaccinia virus Ankara expressing the hemagglutinin gene confers protection against homologous and heterologous H5N1 influenza virus infections in macaques. J Infect Dis 199:405-13.

Kreijtz, J. H., Y. Suezer, G. van Amerongen, G. de Mutsert, B. S. Schnierle, J. M. Wood, T. Kuiken, R. A. Fouchier, J. Lower, A. D. Osterhaus, G. Sutter, and G. F. Rimmelzwaan. 2007. Recombinant modified vaccinia virus Ankara-based vaccine induces protective immunity in mice against infection with influenza virus H5N1. J. Infect. Dis. 195:1598-1606.

Lambert, L. C., and A. S. Fauci. 2010. Influenza Vaccines for the Future. N Engl J Med 363:2036-2044.

Lee, L. Y., L. A. Ha do, C. Simmons, J. M. D. de, N. V. Chau, R. Schumacher, Y. C. Peng, A. J. McMichael, J. J. Farrar, G. L. Smith, A. R. Townsend, B. A. Askonas, S. Rowland-Jones, and T. Dong. 2008. Memory T cells established by seasonal human influenza A infection cross-react with avian influenza A (H5N1) in healthy individuals. J.Clin.Invest 118:3478-3490.

Mackett,M., Smith,G.L., and Moss,B. 1982. Vaccinia virus: a selectable eukaryotic cloning and expression vector. Proc. Natl. Acad. Sci. U. S. A 79:7415-7419.

Mayrhofer, J., S. Coulibaly, A. Hessel, G. W. Holzer, M. Schwendinger, P. Bruhl, M. Gerencer, B. A. Crowe, S. Shuo, W. Hong, Y. J. Tan, B. Dietrich, N. Sabarth, H. Savidis-Dacho, O. Kistner, P. N. Barrett, and F. G. Falkner. 2009. Nonreplicating vaccinia virus vectors expressing the H5 influenza virus hemagglutinin produced in modified Vero cells induce robust protection. J Virol 83:5192-203.

McMichael, A. J., C. A. Michie, F. M. Gotch, G. L. Smith, and B. Moss. 1986. Recognition of influenza A virus nucleoprotein by human cytotoxic T lymphocytes. J Gen Virol 67 (Pt 4):719-26.

Meyer,H., Sutter,G., and Mayr,A. 1991. Mapping of deletions in the genome of the highly attenuated vaccinia virus MVA and their influence on virulence. J Gen. Virol. 72 (Pt 5):1031-1038.

Moss, B., M. W. Carroll, L. S. Wyatt, J. R. Bennink, V. M. Hirsch, S. Goldstein, W. R. Elkins, T. R. Fuerst, J. D. Lifson, M. Piatak, N. P. Restifo, W. Overwijk, R. Chamberlain, S. A. Rosenberg, and G. Sutter. 1996. Host range restricted, non-replicating vaccinia virus vectors as vaccine candidates. Adv Exp Med Biol 397:7-13.

Neirynck, S., T. Deroo, X. Saelens, P. Vanlandschoot, W. M. Jou, and W. Fiers. 1999. A universal influenza A vaccine based on the extracellular domain of the M2 protein. Nat Med 5:1157-63.

Okuno, Y., Y. Isegawa, F. Sasao, and S. Ueda. 1993. A common neutralizing epitope conserved between the hemagglutinins of influenza A virus H1 and H2 strains. J Virol 67:2552-8.

Poon, L. L., Y. H. Leung, J. M. Nicholls, P. Y. Perera, J. H. Lichy, M. Yamamoto, T. A. Waldmann, J. S. Peiris, and L. P. Perera. 2009. Vaccinia virus-based multivalent H5N1 avian influenza vaccines adjuvanted with IL-15 confer sterile cross-clade protection in mice. J Immunol 182:3063-71.

Price, G. E., M. R. Soboleski, C. Y. Lo, J. A. Misplon, C. Pappas, K. V. Houser, T. M. Tumpey, and S. L. Epstein. 2009. Vaccination focusing immunity on conserved antigens protects mice and ferrets against virulent H1N1 and H5N1 influenza A viruses. Vaccine 27:6512-21.

Price, G. E., M. R. Soboleski, C. Y. Lo, J. A. Misplon, M. R. Quirion, K. V. Houser, M. B. Pearce, C. Pappas, T. M. Tumpey, and S. L. Epstein. 2010. Single-dose mucosal immunization with a candidate universal influenza vaccine provides rapid protection from virulent H5N1, H3N2 and H1N1 viruses. PLoS One 5:e13162.

Pushko, P., T. M. Tumpey, F. Bu, J. Knell, R. Robinson, and G. Smith. 2005. Influenza virus-like particles comprised of the HA, NA, and M1 proteins of H9N2 influenza virus induce protective immune responses in BALB/c mice. Vaccine 23:5751-9.

Ricci et al., 2011. Virology Journal , 8:529.

Rimmelzwaan, G. F., and G. Sutter. 2009. Candidate influenza vaccines based on recombinant modified vaccinia virus Ankara. Expert Rev Vaccines 8:447-54.

Sanchez-Fauquier, A., N. Villanueva, and J. A. Melero. 1987. Isolation of cross-reactive, subtype-specific monoclonal antibodies against influenza virus HA1 and HA2 hemagglutinin subunits. Arch Virol 97:251-65.

Scheiflinger et al, 1998. Arch. Virol. 143, 467-474.

Schotsaert, M., M. De Filette, W. Fiers, and X. Saelens. 2009. Universal M2 ectodomain-based influenza A vaccines: preclinical and clinical developments. Expert Rev Vaccines 8:499-508.

Slavik,I., Ciampor,F., and Mayer,V. 1983. Optimalized conditions of tick-borne encephalitis virus production in vitro. Acta Virol. 27:97-104.

Smith, G. L., J. Z. Levin, P. Palese, and B. Moss. 1987. Synthesis and cellular location of the ten influenza polypeptides individually expressed by recombinant vaccinia viruses. Virology 160:336-45.

Song, J. M., J. Hossain, D. G. Yoo, A. S. Lipatov, C. T. Davis, F. S. Quan, L. M. Chen, R. J. Hogan, R. O. Donis, R. W. Compans, and S. M. Kang. 2010. Protective immunity against H5N1 influenza virus by a single dose vaccination with virus-like particles. Virology 405:165-75.

Steel, J., A. C. Lowen, T. Wang, M. Yondola, Q. Gao, K. Haye, A. Garcia-Sastre, and P. Palese. 2010. Influenza virus vaccine based on the conserved hemagglutinin stalk domain. MBio 1.

Sui, J., W. C. Hwang, S. Perez, G. Wei, D. Aird, L. M. Chen, E. Santelli, B. Stec, G. Cadwell, M. Ali, H. Wan, A. Murakami, A. Yammanuru, T. Han, N. J. Cox, L. A. Bankston, R. O. Donis, R. C. Liddington, and W. A. Marasco. 2009. Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. Nat Struct Mol Biol 16:265-73.

Throsby, M., E. van den Brink, M. Jongeneelen, L. L. Poon, P. Alard, L. Cornelissen, A. Bakker, F. Cox, E. van Deventer, Y. Guan, J. Cinatl, J. ter Meulen, I. Lasters, R. Carsetti, M. Peiris, J. de Kruif, and J. Goudsmit. 2008. Heterosubtypic neutralizing monoclonal antibodies cross-protective against H5N1 and H1N1 recovered from human IgM+ memory B cells. PLoS One 3:e3942.

Tykodi,S.S. and Thompson,J.A. 2008. Development of modified vaccinia Ankara-5T4 as specific immunotherapy for advanced human cancer. Expert. Opin. Biol. Ther 8:1947-1953.

Ulmer, J. B., J. J. Donnelly, S. E. Parker, G. H. Rhodes, P. L. Felgner, V. J. Dwarki, S. H. Gromkowski, R. R. Deck, C. M. DeWitt, A. Friedman, and et al. 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science 259:1745-9.

Wyatt, L. S., S. T. Shors, B. R. Murphy, and B. Moss. 1996. Development of a replication-deficient recombinant vaccinia virus vaccine effective against parainfluenza virus 3 infection in an animal model. Vaccine 14:1451-8.

Yewdell, J. W., J. R. Bennink, G. L. Smith, and B. Moss. 1985. Influenza A virus nucleoprotein is a major target antigen for cross-reactive anti-influenza A virus cytotoxic T lymphocytes. Proc Natl Acad Sci U S A 82:1785-9.

Zhao, G., Y. Lin, L. Du, J. Guan, S. Sun, H. Sui, Z. Kou, C. C. Chan, Y. Guo, S. Jiang, B. J. Zheng, and Y. Zhou. 2010. An M2e-based multiple antigenic peptide vaccine protects mice from lethal challenge with divergent H5N1 influenza viruses. Virol J 7:9.

Zhao, G., S. Sun, L. Du, W. Xiao, Z. Ru, Z. Kou, Y. Guo, H. Yu, S. Jiang, Y. Lone, B. J. Zheng, and Y. Zhou. 2010a. An H5N1 M2e-based multiple antigenic peptide vaccine confers heterosubtypic protection from lethal infection with pandemic 2009 H1N1 virus. Virol J 7:151.

Zhou, D., T. L. Wu, M. O. Lasaro, B. P. Latimer, E. M. Parzych, A. Bian, Y. Li, H. Li, J. Erikson, Z. Xiang, and H. C. Ertl. 2010b. A universal influenza a vaccine based on adenovirus expressing matrix-2 ectodomain and nucleoprotein protects mice from lethal challenge. Mol Ther 18:2182-9.

CLAIMS

We claim:

1. A recombinant vaccinia virus comprising a gene cassette encoding a fusion protein comprising at least one influenza A M2 extracellular domain (M2e) polypeptide inserted in an influenza A headless hemagglutinin (hlHA) polypeptide.

2. The recombinant vaccinia virus of claim 1 wherein the fusion protein comprises the hlHA/M2e fusion protein amino acid sequence set out in SEQ ID NO: 2.

3. The recombinant vaccinia virus of claim 1 wherein the fusion protein comprises amino acids of the VN/1203 HA amino acid sequence set out in SEQ ID NO: 3.

4. The recombinant vaccinia virus of claim 3 wherein the fusion protein comprises HA1 amino acids 17-58 of SEQ ID NO: 3, a peptide linker, at least one M2e polypeptide, a peptide linker, HA1 amino acids 290-343 of SEQ ID NO: 3 and HA2 amino acids 344-568 of SEQ ID NO: 3.

5. The recombinant vaccinia virus of claim 4 wherein the peptide linkers linking the HA amino acids and M2e amino acids comprise the amino acids GGG set out in SEQ ID NO: 4.

6. The recombinant vaccinia virus of claim 1 wherein the M2e polypeptide comprises the H5N1 M2e amino acid sequence set out in SEQ ID NO: 5.

7. The recombinant vaccinia virus of claim 1 wherein the M2e polypeptide comprises the H1N1 M2e amino acid sequence set out in SEQ ID NO: 6.

8. The recombinant vaccinia virus of claim 1 wherein the M2e polypeptide comprises the H9N2 M2e amino acid sequence set out in SEQ ID NO: 7.

9. The recombinant vaccinia virus of claim 1 wherein the M2e polypeptide comprises the H7N2 M2e amino acid sequence set out in SEQ ID NO: 8.

10. The recombinant vaccinia virus of claim 1, 3, 4, 5, 6, 7, 8 or 9 wherein the fusion protein comprises more than one M2e polypeptide and the M2e polypeptides are linked by a peptide linker.

11. The recombinant vaccinia virus of claim 10 wherein the peptide linker linking the M2e polypeptides comprises the amino acids GSAGSA set out in SEQ ID NO: 9.

12. The recombinant vaccinia virus of any of claims 1-11 wherein expression of the hlHA/M2e fusion protein from the gene cassette is under the control of an mH5 promoter.

13. The recombinant vaccinia virus of any of claims 1-11 wherein expression of the hlHA/M2e fusion protein from the gene cassette is under the control of a selP promoter.

14. A recombinant vaccinia virus comprising the gene cassette set out in SEQ ID NO: 1.

15. The recombinant vaccinia virus of any one of claims 1-14 further comprising a gene cassette encoding an influenza A matrix protein 1 (M1) and a gene cassette encoding an influenza A nucleoprotein (NP).

16. The recombinant vaccinia virus of claim 15 wherein the M1 protein comprises the amino acid sequence set out in SEQ ID NO: 11.

17. The recombinant vaccinia virus of claim 16 wherein the M1 protein is encoded by the nucleotide sequence set out in SEQ ID NO: 10.

18. The recombinant vaccinia virus of claim 15 wherein the NP comprises the amino acid sequence set out in SEQ ID NO: 13.

19. The recombinant vaccinia virus of claim 18 wherein the NP is encoded by the nucleotide sequence set out in SEQ ID NO: 12.

20. The recombinant vaccinia virus of any of claims 1-19 wherein the vaccinia virus is modified vaccinica virus Ankara (MVA), defective vaccinia Lister (dVV), MVA-575, MVA-BN, MVA-F6 or MVA-M4.

21. The recombinant vaccinia virus of any of claims 1-19 wherein the vaccinia virus is MVA.

22. The recombinant MVA of claim 21 wherein the hlHA/M2e fusion protein gene cassette is inserted in the MVA in the deletion I region, the deletion II region, the deletion III region, the deletion IV region, the thymidine kinase locus, the D4/5 intergenic region or the HA locus.

23. The recombinant MVA of claim 21 or 22 wherein the hlHA/M2e fusion protein gene cassette is inserted in the MVA in the D4/5 intergenic region.

24. A pharmaceutical composition comprising the recombinant MVA of any one of claims 1-23.

25. A pharmaceutical composition comprising a recombinant MVA comprising a gene cassette encoding the hlHA/M2e fusion protein amino acid sequence set out in SEQ ID NO: 2.

26. A pharmaceutical composition comprising a recombinant MVA comprising the gene cassette set out in SEQ ID NO: 1.

27. A method of inducing a heterosubtypic immune response to influenza A virus in an individual comprising administering a pharmaceutical composition comprising the recombinant MVA of any one of claims 1-23 to the individual.

28. A pharmaceutical composition for use in inducing a heterosubtypic immune response to influenza A virus in an individual, the method comprising the step of administering a pharmaceutical composition comprising the recombinant MVA of any one of claims 1-23 to the individual.

29. A method of producing a recombinant MVA expressing the hlHA/M2e fusion protein gene cassette set out in SEQ ID NO: 1 comprising the steps of:

a) infecting primary chicken embryo cells or a permanent cell line with MVA,

b) transfecting the infected cells with a plasmid comprising the hlHA/M2e fusion protein gene cassette set out in SEQ ID NO: 1 and comprising DNA flanking the gene cassette that is homologous to a non-essential region of the MVA genome,

c) growing the cells to allow the plasmid to recombine with the MVA genome during replication of the MVA in the infected cells thereby inserting the hlHA/M2e fusion protein gene cassette into the MVA genome in the non-essential region, and

d) obtaining the recombinant MVA produced.

30. The method of claim 29 wherein the non-essential MVA region is the deletion I region, the deletion II region, the deletion III region, the deletion IV region, the thymidine kinase locus, the D4/5 intergenic region, or the HA locus.

31. The method of claim 30 wherein the non-essential MVA region is the D4/5 intergenic region.

32. A recombinant vaccinia virus comprising a first gene cassette encoding a fusion protein comprising at least one influenza A M2 extracellular domain (M2e) polypeptide inserted in an influenza A headless hemagglutinin (hlHA) polypeptide and a second gene cassette encoding influenza A nucleoprotein (NP).

33. The recombinant vaccinia virus of claim 32 wherein the fusion protein comprises the hlHA/M2e fusion protein amino acid sequence set out in SEQ ID NO: 2.

34. The recombinant vaccinia virus of claim 32 wherein the fusion protein comprises amino acids of the VN/1203 HA amino acid sequence set out in SEQ ID NO: 3.

35. The recombinant vaccinia virus of claim 34 wherein the fusion protein comprises HA1 amino acids 17-58 of SEQ ID NO: 3, a peptide linker, at least one M2e polypeptide, a peptide linker, HA1 amino acids 290-343 of SEQ ID NO: 3 and HA2 amino acids 344-568 of SEQ ID NO: 3.

36. The recombinant vaccinia virus of claim 35 wherein the peptide linkers linking the HA amino acids and M2e amino acids comprise the amino acids GGG set out in SEQ ID NO: 4.

37. The recombinant vaccinia virus of claim 32 wherein the M2e polypeptide comprises the H5N1 M2e amino acid sequence set out in SEQ ID NO: 5.

38. The recombinant vaccinia virus of claim 32 wherein the M2e polypeptide comprises the H1N1 M2e amino acid sequence set out in SEQ ID NO: 6.

39. The recombinant vaccinia virus of claim 32 wherein the M2e polypeptide comprises the H9N2 M2e amino acid sequence set out in SEQ ID NO: 7.

40. The recombinant vaccinia virus of claim 32 wherein the M2e polypeptide comprises the H7N2 M2e amino acid sequence set out in SEQ ID NO: 8.

41. The recombinant vaccinia virus of claim 32, 34, 35, 36, 37, 38, 39 or 40 wherein the fusion protein comprises more than one M2e polypeptide and the M2e polypeptides are linked by a peptide linker.

42. The recombinant vaccinia virus of claim 41 wherein the peptide linker linking the M2e polypeptides comprises the amino acids GSAGSA set out in SEQ ID NO: 9.

43. The recombinant vaccinia virus of claim 32 wherein the NP comprises the amino acid sequence set out in SEQ ID NO: 13.

44. The recombinant vaccinia virus of any of claims 32-43 wherein expression of the hlHA/M2e fusion protein from the first gene cassette is under the control of an mH5 promoter or a selP promoter.

45. The recombinant vaccinia virus of claim 44 wherein expression of the hlHA/M2e fusion protein from the first gene cassette is under the control of an mH5 promoter

46. The recombinant vaccinia virus of claim 44 wherein expression of the hlHA/M2e fusion protein from the first gene cassette is under the control of a selP promoter.

47. The recombinant vaccinia virus of any of claims 32-43 wherein expression of NP from the second gene cassette is under the control of an mH5 promoter or selP promoter.

48. The recombinant vaccinia virus of claim 47 wherein expression of NP from the second gene cassette is under the control of an mH5 promoter

49. The recombinant vaccinia virus of claim 47 wherein expression of NP from the second gene cassette is under the control of a selP promoter.

50. The recombinant vaccinia virus of any of claims 32-43 wherein expression of the hlHA/M2e fusion protein from the first gene cassette and the expression of NP from the second gene cassette are under the control of mH5 promoters.

51. A recombinant vaccinia virus comprising a first gene cassette set out in SEQ ID NO: 1 and a second gene cassette set out in SEQ ID NO: 12.

52. The recombinant vaccinia virus of any of claims 32-51 wherein the vaccinia virus is modified vaccinica virus Ankara (MVA), defective vaccinia Lister (dVV), MVA-575, MVA-BN, MVA-F6 or MVA-M4.

53. The recombinant vaccinia virus of any of claims 32-51 wherein the vaccinia virus is MVA.

54. The recombinant MVA of claim 53 wherein the first gene cassette is inserted in the MVA in the deletion I region, the deletion II region, the deletion III region, the deletion IV region, the thymidine kinase locus, the D4/5 intergenic region or the HA locus.

55. The recombinant MVA of claim 53 wherein the second gene cassette is inserted in the MVA in the deletion I region, the deletion II region, the deletion III region, the deletion IV region, the thymidine kinase locus, the D4/5 intergenic region or the HA locus.

56. The recombinant MVA of any one of claims 32-53 wherein the first gene cassette is inserted in the MVA in the D4/5 intergenic region.

57. The recombinant MVA of any one of claims 32-53 wherein the second gene cassette is inserted in the MVA in the deletion III region.

59. The recombinant MVA of any one of claims 32-53 wherein the first gene cassette is inserted in the MVA in the D4/5 intergenic region and the second gene cassette is inserted in the MVA in the deletion III region

60. A pharmaceutical composition comprising the recombinant MVA of any one of claims 32-59.

61. A pharmaceutical composition comprising a recombinant MVA comprising a first gene cassette encoding the hlHA/M2e fusion protein amino acid sequence set out in SEQ ID NO: 2 and a second gene cassette encoding influenza A nucleoprotein (NP) set out in SEQ ID NO: 13.

62. A pharmaceutical composition comprising a recombinant MVA comprising a hlHA/M2e fusion protein first gene cassette set out in SEQ ID NO: 1 and an NP second gene cassette set out in SEQ ID NO: 12.

63. A method of inducing a heterosubtypic immune response to influenza A viruses in an individual comprising administering a pharmaceutical composition comprising the recombinant MVA of any one of claims 32-59 to the individual.

64. A pharmaceutical composition for use in inducing a heterosubtypic immune response to influenza A viruses in an individual, the method comprising the step of administering a pharmaceutical composition comprising the recombinant MVA of any one of claims 32-59 to the individual.

65. A method of producing a recombinant MVA expressing a hlHA/M2e fusion protein first gene cassette set out in SEQ ID NO: 1 and a NP second gene cassette set out in SEQ ID NO: 12 comprising the steps of:

a) infecting primary chicken embryo cells or a permanent cell line with MVA,

b) transfecting the infected cells with a plasmid comprising the hlHA/M2e fusion protein gene cassette set out in SEQ ID NO: 1 and comprising DNA flanking the gene cassette that is homologous to a first non-essential region of the MVA genome,

c) growing the cells to allow the plasmid to recombine with the MVA genome during replication of the MVA in the infected cells thereby inserting the hlHA/M2e fusion protein gene cassette into the MVA genome in the first non-essential region,

d) obtaining the recombinant MVA produced;

e) infecting primary chicken embryo cells or a permanent avian cell line with the MVA of step d),

f) transfecting the infected cells of step e) with a plasmid comprising the NP gene cassette set out in SEQ ID NO: 12 and comprising DNA flanking the gene cassette that is homologous to a second non-essential region of the MVA genome, wherein the second non-essential region is not the same region as the first non-essential region,

g) growing the cells to allow the plasmid to recombine with the MVA genome during replication of the MVA in the infected cells thereby inserting the NP gene cassette into the MVA genome in the second non-essential region, and

h) obtaining the recombinant MVA produced.

66. The method of claim 65 wherein the non-essential MVA regions are the deletion I region, the deletion II region, the deletion III region, the deletion IV region, the thymidine kinase locus, the D4/5 intergenic region, or the HA locus.

67. The method of claim 65 wherein the first non-essential MVA region is the D4/5 intergenic region.

68. The method of claim 65 wherein the second non-essential MVA region is the deletion III region.

69. The method of claim 65 wherein the first non-essential MVA region is the D4/5 intergenic region and the second non-essential MVA region is the deletion III region.

70. A recombinant vaccinia virus comprising a first gene cassette encoding an influenza A headless hemagglutinin (hlHA) polypeptide and a second gene cassette encoding influenza A nucleoprotein (NP).

71. The recombinant vaccinia virus of claim 60 wherein the hlHA polypeptide comprises the amino acid sequence set out in SEQ ID NO: 15.

72. The recombinant vaccinia virus of claim 60 wherein the NP comprises the amino acid sequence set out in SEQ ID NO: 13.

73. The recombinant vaccinia virus of any of claims 70-72 wherein expression of the hlHA polypeptide from the first gene cassette is under the control of an mH5 promoter or a selP promoter.

74. The recombinant vaccinia virus of claim 73 wherein expression of the hlHA polypeptide from the first gene cassette is under the control of an mH5 promoter

75. The recombinant vaccinia virus of claim 73 wherein expression of the hlHA polypeptide from the first gene cassette is under the control of a selP promoter.

76. The recombinant vaccinia virus of any of claims 70-72 wherein expression of NP from the second gene cassette is under the control of an mH5 promoter or selP promoter.

77. The recombinant vaccinia virus of claim 76 wherein expression of NP from the second gene cassette is under the control of an mH5 promoter.

78. The recombinant vaccinia virus of claim 76 wherein expression of NP from the second gene cassette is under the control of a selP promoter.

79. The recombinant vaccinia virus of any of claims 70-72 wherein expression of the hlHA polypeptide from the first gene cassette and the expression of NP from the second gene cassette are under the control of mH5 promoters.

80. A recombinant vaccinia virus comprising a hlHA first gene cassette set out in SEQ ID NO: 14 and an NP second gene cassette set out in SEQ ID NO: 12.

81. The recombinant vaccinia virus of any one of claims 70-80 further comprising a third gene cassette encoding an influenza A M1.

82. The recombinant vaccinia virus of claim 15 wherein the M1 protein comprises the amino acid sequence set out in SEQ ID NO: 11.

83. The recombinant vaccinia virus of claim 82 wherein the M1 protein is encoded by the nucleotide sequence set out in SEQ ID NO: 10.

84. The recombinant vaccinia virus of any of claims 70-83 wherein the vaccinia virus is modified vaccinica virus Ankara (MVA), defective vaccinia Lister (dVV), MVA-575, MVA-BN, MVA-F6 or MVA-M4.

85. The recombinant vaccinia virus of any of claims 70-83 wherein the vaccinia virus is MVA.

86. The recombinant MVA of claim 85 wherein the first gene cassette is inserted in the MVA in the deletion I region, the deletion II region, the deletion III region, the deletion IV region, the thymidine kinase locus, the D4/5 intergenic region or the HA locus.

87. The recombinant MVA of claims 85 or 86 wherein the second gene cassette is inserted in the MVA in the deletion I region, the deletion II region, the deletion III region, the deletion IV region, the thymidine kinase locus, the D4/5 intergenic region or the HA locus.

88. The recombinant MVA of claim 85 wherein the first gene cassette is inserted in the MVA in the D4/5 intergenic region.

89. The recombinant MVA of claim 85 wherein the second gene cassette is inserted in the MVA in the deletion III region.

90. The recombinant MVA of claim 85 wherein the first and third gene cassettes are inserted in the MVA in the D4/5 intergenic region and the second gene cassette is inserted in the MVA in the deletion III region.

91. A pharmaceutical composition comprising the recombinant MVA of any one of claims 70-90.

92. A pharmaceutical composition comprising a recombinant MVA comprising a first gene cassette encoding the headless HA (hlHA) amino acid sequence set out in SEQ ID NO: 15 and a second gene cassette encoding influenza A nucleoprotein (NP) set out in SEQ ID NO: 13.

93. A pharmaceutical composition comprising a recombinant MVA comprising a hlHA first gene cassette set out in SEQ ID NO: 14 and an NP second gene cassette set out in SEQ ID NO: 12.

94. A pharmaceutical composition comprising a recombinant MVA comprising a hlHA first gene cassette set out in SEQ ID NO: 14, an M1 second gene cassette set out in SEQ ID NO: 10 and an NP third gene cassette set out in SEQ ID NO: 12.

95. A method of inducing a heterosubtypic immune response to influenza A viruses in an individual comprising administering a pharmaceutical composition comprising the recombinant MVA of any one of claims 70-90 to the individual.

96. A pharmaceutical composition for use in inducing a heterosubtypic immune response to influenza A viruses in an individual, the method comprising the step of administering a pharmaceutical composition comprising the recombinant MVA of any one of claims 70-90 to the individual.

97. A method of producing a recombinant MVA expressing a hlHA first gene cassette set out in SEQ ID NO: 14 and a NP second gene cassette set out in SEQ ID NO: 12 comprising the steps of:

a) infecting primary chicken embryo cells or a permanent avian cell line with MVA,

b) transfecting the infected cells with a plasmid comprising the headless HA/M2e fusion protein gene cassette set out in SEQ ID NO: 14 and comprising DNA flanking the gene cassette that is homologous to a first non-essential region of the MVA genome,

c) growing the cells to allow the plasmid to recombine with the MVA genome during replication of the MVA in the infected cells thereby inserting the headless HA/M2e fusion protein gene cassette into the MVA genome in the first non-essential region,

d) obtaining the recombinant MVA produced;

e) infecting primary chicken embryo cells or a permanent avian cell line with the MVA of step d),

f) transfecting the infected cells of step e) with a plasmid comprising the NP gene cassette set out in SEQ ID NO: 12 and comprising DNA flanking the gene cassette that is homologous to a second non-essential region of the MVA genome, wherein the second non-essential region is not the same region as the first non-essential region,

g) growing the cells to allow the plasmid to recombine with the MVA genome during replication of the MVA in the infected cells thereby inserting the NP gene cassette into the MVA genome in the second non-essential region, and

h) obtaining the recombinant MVA produced.

98. The method of claim 97 wherein the non-essential MVA regions are the deletion I region, the deletion II region, the deletion III region, the deletion IV region, the thymidine kinase locus, the D4/5 intergenic region, or the HA locus.

99. The method of claim 97 wherein the first non-essential MVA region is the D4/5 intergenic region.

100. The method of claim 97 wherein the second non-essential MVA region is the deletion III region.

101. The method of claim 97 wherein the first non-essential MVA region is the D4/5 intergenic region and the second non-essential MVA region is the deletion III region.

FIGURE 1

MEKIVLLFAIVSLVKSDQICIGYHANNSTEQVDTI
MEKNVTVTHAQDILEKKHNGKLCGGGGCNTKCQ
TPMGAINSSMPFHNIHPLTIGECPKYVKSNRLVLAT
GLRNSPQRERRRKKRGLFGAIAGFIEGGWQGMVD
GWYGYHHSNEQGSGYAADKESTQKAIDGVTNKV
NSIIDKMNTQFEAVGREFNNLERRIENLNKKMEDG
FLDVWTYNAELLVLMENERTLDFHDSNVKNLYDK
VRLQLRDNAKELGNGCFEFYHKCDNECMESVRNG
TYDYPQYSEEARLKREEISGVKLESIGIYQILSIYST
VASSLALAIMVAGLSLWMCSNGSLQCRICI

FIGURE 2

ATGGAGAAAATAGTGCTTCTTTTTGCAATAGTCAGTCT
TGTTAAAAGTGATCAGATTTGCATTGGTTACCATGCAA
ACAACTCGACAGAGCAGGTTGACACAATAATGGAAA
AGAACGTTACTGTTACACATGCCCAAGACATACTGGA
AAAGAAACACAACGGGAAGCTCTGCGGAGGAGGAGG
ATGCAACACCAAGTGTCAAACTCCAATGGGGGCGATA
AACTCTAGCATGCCATTCCACAATATACACCCTCTCAC
CATTGGGGAATGCCCCAAATATGTGAAATCAAACAGA
TTAGTCCTTGCGACTGGGCTCAGAAATAGCCCTCAAA
GAGAGAGAAGAAGAAAAAAGAGAGGATTATTTGGAG
CTATAGCAGGTTTTATAGAGGGAGGATGGCAGGGAAT
GGTAGATGGTTGGTATGGGTACCACCATAGCAATGAG
CAGGGGAGTGGGTACGCTGCAGACAAAGAATCCACTC
AAAAGGCAATAGATGGAGTCACCAATAAGGTCAACTC
GATCATTGACAAAATGAACACTCAGTTTGAGGCCGTT
GGAAGGGAATTTAACAACTTAGAAAGGAGAATAGAG
AATTTAAACAAGAAGATGGAAGACGGGTTCCTAGATG
TCTGGACTTATAATGCTGAACTTCTGGTTCTCATGGAA
AATGAGAGAACTCTAGACTTTCATGACTCAAATGTCA
AGAACCTTTACGACAAGGTCCGACTACAGCTTAGGGA
TAATGCAAAGGAGCTGGGTAACGGTTGTTTCGAGTTC
TATCATAAATGTGATAATGAATGTATGGAAAGTGTAA
GAAATGGAACGTATGACTACCCGCAGTATTCAGAAGA
AGCGAGACTAAAAAGAGAGGAAATAAGTGGAGTAAA
ATTGGAATCAATAGGAATTTACCAAATACTGTCAATTT
ATTCTACAGTGGCGAGTTCCCTAGCACTGGCAATCAT
GGTAGCTGGTCTATCCTTATGGATGTGCTCCAATGGAT
CGTTACAATGCAGAATTTGCATTTAA

FIGURE 3

MEKIVLLFAIVSLVKS*DQICIGYHANNSTEQVDTIME*
*KNVTVTHAQDILEKKHNGKLC*GGGSLLTEVETPTRN
EWECRCSDSSDGSAGSASLLTEVETPIRNEWGCRC
NDSSDGSAGSASLLTEVETPTRNGWECKCSDSSDG
SAGSASLLTEVETPIRKGWECNCSDSSDGGG*CNTK*
CQTPMGAINSSMPFHNIHPLTIGECPKYVKSNRLVLAT
*GLRNSPQRERRR*KKRGLFGAIAGFIEGGWQGMVDG
WYGYHHSNEQGSGYAADKESTQKAIDGVTNKVN
SIIDKMNTQFEAVGREFNNLERRIENLNKKMEDGF
LDVWTYNAELLVLMENERTLDFHDSNVKNLYDK
VRLQLRDNAKELGNGCFEFYHKCDNECMESVRNG
TYDYPQYSEEARLKREEISGVKLESIGIYQILSIYST
VASSLALAIMVAGLSLWMCSNGSLQCRICI

FIGURE 4

ATGGAGAAAATAGTGCTTCTTTTTGCAATAGTCAGTCTTGTTA
AAAGTGATCAGATTTGCATTGGTTACCATGCAAACAACTCGA
CAGAGCAGGTTGACACAATAATGGAAAAGAACGTTACTGTT
ACACATGCCCAAGACATACTGGAAAAGAAACACAACGGGAA
GCTCTGCGGAGGAGGAAGTCTTCTAACCGAGGTCGAAACGCC
TACCAGAAACGAATGGGAGTGCAGATGCAGCGATTCAAGTG
ATGGAAGTGCAGGATCAGCGAGTCTTCTAACCGAGGTCGAA
ACGCCTATCAGAAACGAATGGGGGTGCAGATGCAACGATTC
AAGTGATGGAAGTGCAGGATCAGCGAGTCTTCTAACCGAGGT
CGAAACGCCTACCAGAAACGGATGGGAGTGCAAATGCAGCG
ATTCAAGTGATGGAAGTGCAGGATCAGCGAGTCTTCTAACCG
AGGTCGAAACGCCTATCAGAAAAGGATGGGAGTGCAACTGC
AGCGATTCAAGTGATGGAGGAGGATGCAACACCAAGTGTCA
AACTCCAATGGGGGCGATAAACTCTAGCATGCCATTCCACAA
TATACACCCTCTCACCATTGGGGAATGCCCCAAATATGTGAA
ATCAAACAGATTAGTCCTTGCGACTGGGCTCAGAAATAGCCC
TCAAAGAGAGAGAAGAAGAAAAAAGAGAGGATTATTTGGAG
CTATAGCAGGTTTTATAGAGGGAGGATGGCAGGGAATGGTA
GATGGTTGGTATGGGTACCACCATAGCAATGAGCAGGGGAG
TGGGTACGCTGCAGACAAAGAATCCACTCAAAAGGCAATAG
ATGGAGTCACCAATAAGGTCAACTCGATCATTGACAAAATGA
ACACTCAGTTTGAGGCCGTTGGAAGGGAATTTAACAACTTAG
AAAGGAGAATAGAGAATTTAAACAAGAAGATGGAAGACGGG
TTCCTAGATGTCTGGACTTATAATGCTGAACTTCTGGTTCTCA
TGGAAAATGAGAGAACTCTAGACTTTCATGACTCAAATGTCA
AGAACCTTTACGACAAGGTCCGACTACAGCTTAGGGATAATG
CAAAGGAGCTGGGTAACGGTTGTTTCGAGTTCTATCATAAAT
GTGATAATGAATGTATGGAAAGTGTAAGAAATGGAACGTAT
GACTACCCGCAGTATTCAGAAGAAGCGAGACTAAAAAGAGA
GGAAATAAGTGGAGTAAAATTGGAATCAATAGGAATTTACC
AAATACTGTCAATTTATTCTACAGTGGCGAGTTCCCTAGCACT
GGCAATCATGGTAGCTGGTCTATCCTTATGGATGTGCTCCAA
TGGATCGTTACAATGCAGAATTTGCATTTAA

FIGURE 5

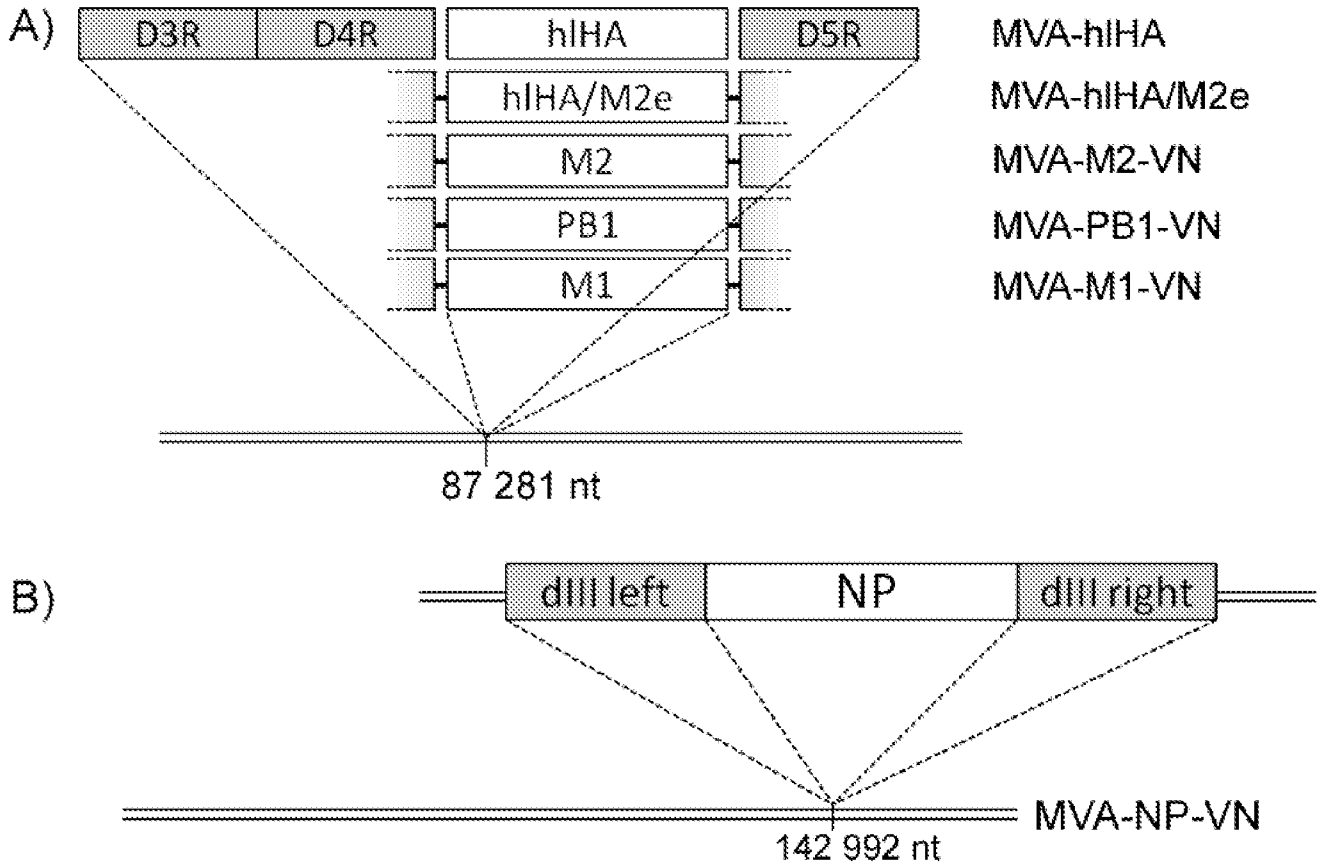
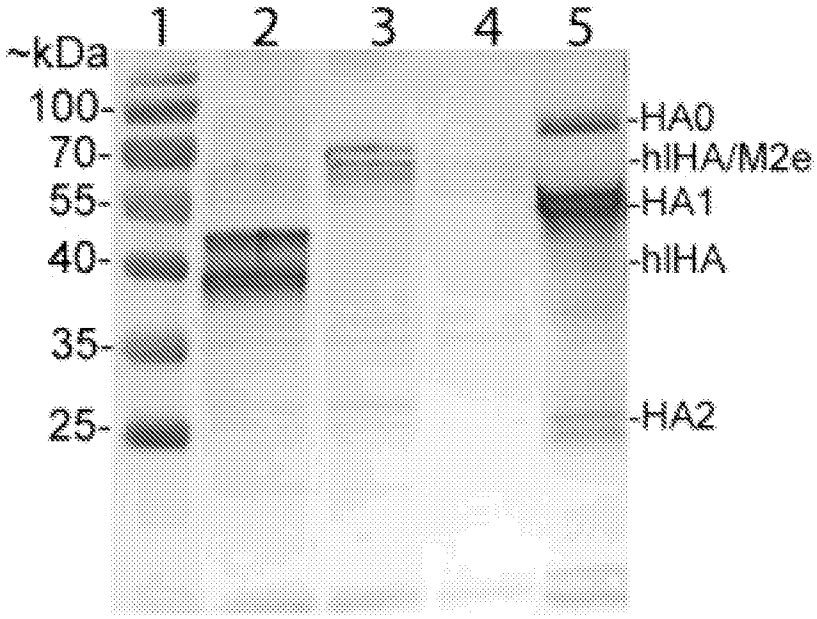

A)
D3R
D4R
hlHA
D5R
hlHA/M2e
M2
PB1
M1
MVA-hlHA
MVA-hlHA/M2e
MVA-M2-VN
MVA-PB1-VN
MVA-M1-VN
87 281 nt
B)
dIII left
NP
dIII right
MVA-NP-VN
142 992 nt

FIGURE 6

A


1
2
3
4
5
~kDa
100-
70-
55-
40-
35-
25-
-HA0
-hlHA/M2e
-HA1
-hlHA
-HA2

B

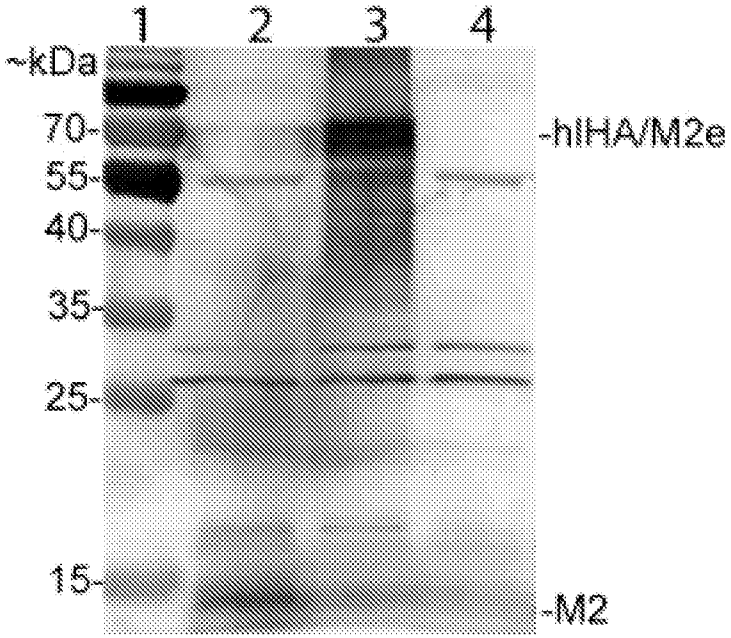

1
2
3
4
~kDa
70-
55-
40-
35-
25-
15-
-hlHA/M2e
-M2

FIGURE 7

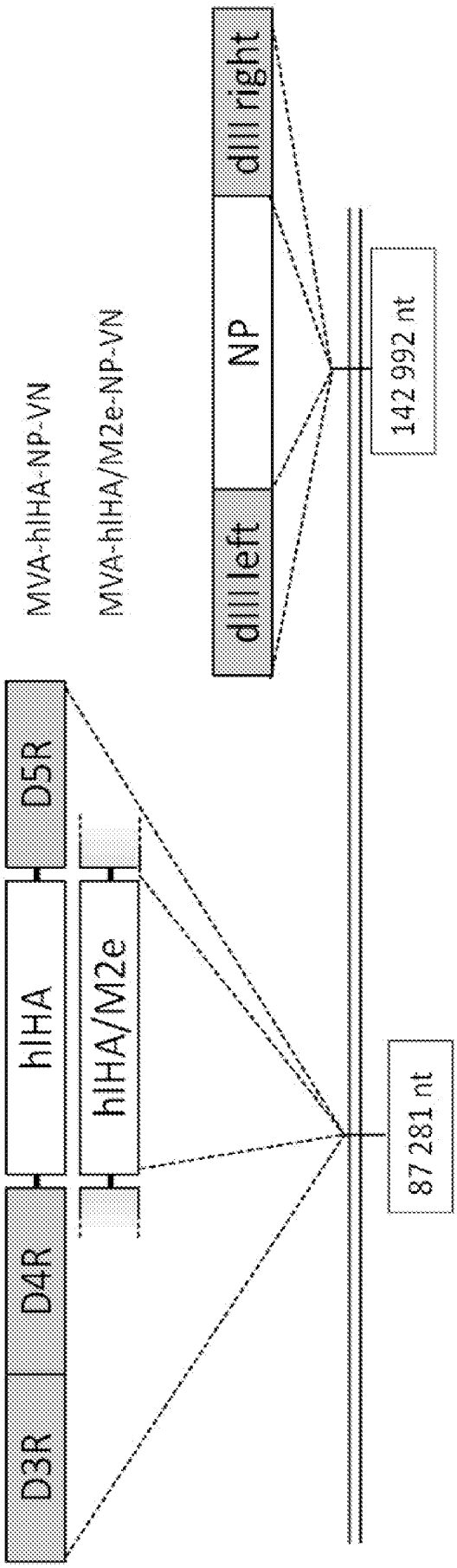
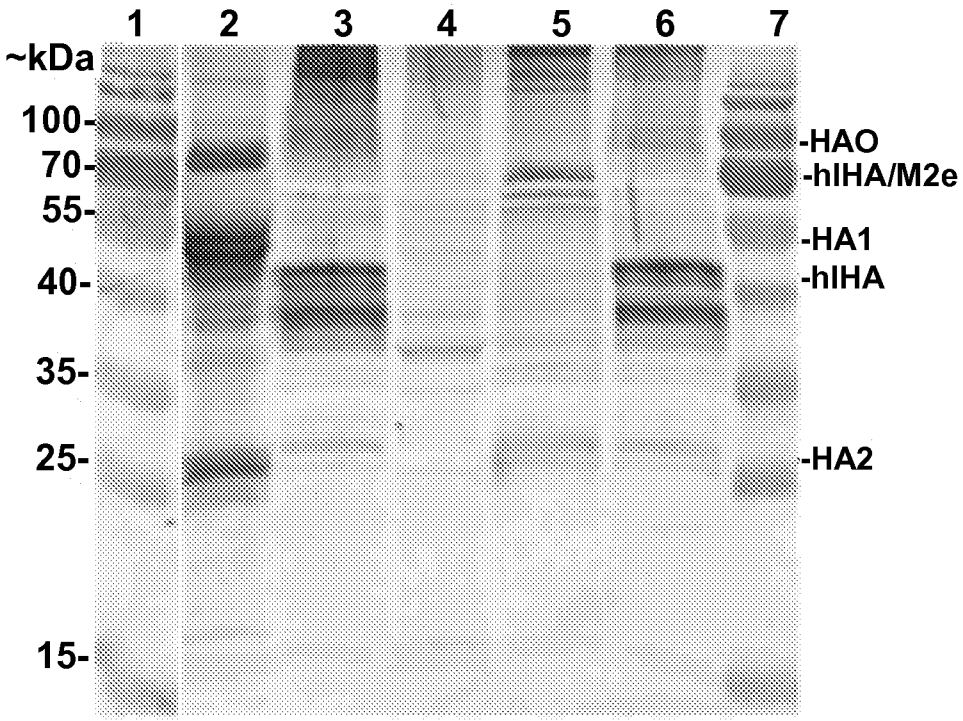

MVA-hlHA-NP-VN
MVA-hlHA/M2e-NP-VN
D3R
D4R
D5R
hlHA
hlHA/M2e
dIII left
NP
dIII right
87 281 nt
142 992 nt

FIGURE 8

A


1
2
3
4
5
6
7
~kDa
100-
70-
55-
40-
35-
25-
15-
-HAO
-hIHA/M2e
-HA1
-hIHA
-HA2

B

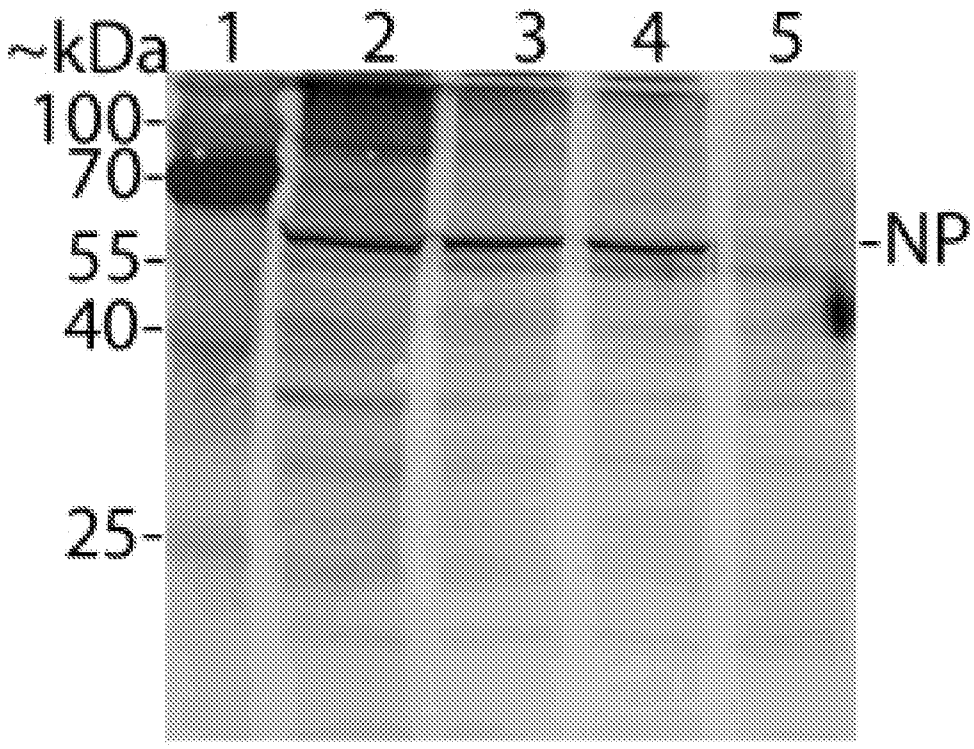

~kDa
1
2
3
4
5
100-
70-
55-
40-
25-
-NP

FIGURE 9

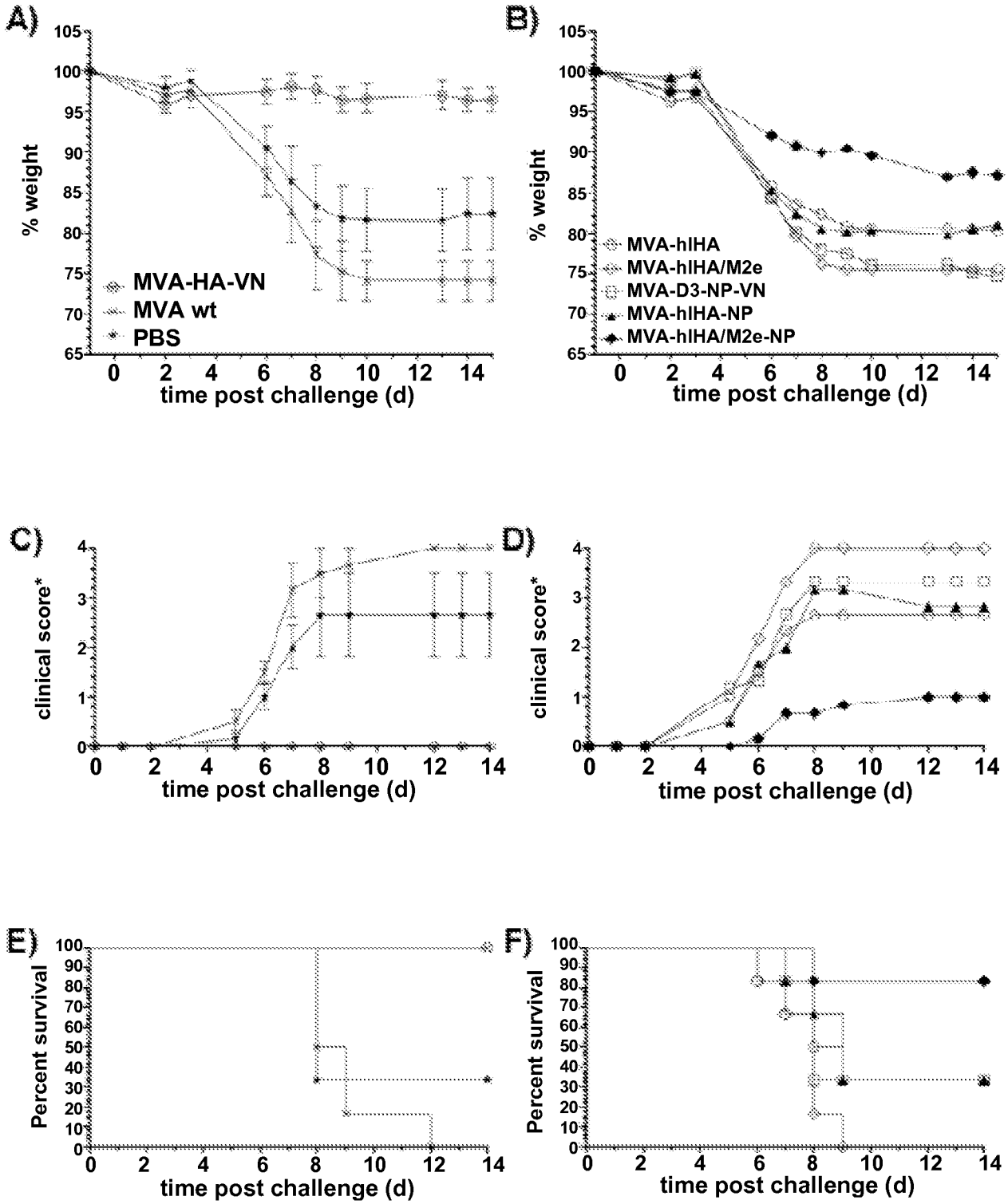

A)
% weight
time post challenge (d)
MVA-HA-VN
MVA wt
PBS
B)
% weight
time post challenge (d)
MVA-hIHA
MVA-hIHA/M2e
MVA-D3-NP-VN
MVA-hIHA-NP
MVA-hIHA/M2e-NP
C)
clinical score*
time post challenge (d)
D)
clinical score*
time post challenge (d)
E)
Percent survival
time post challenge (d)
F)
Percent survival
time post challenge (d)

FIGURE 10

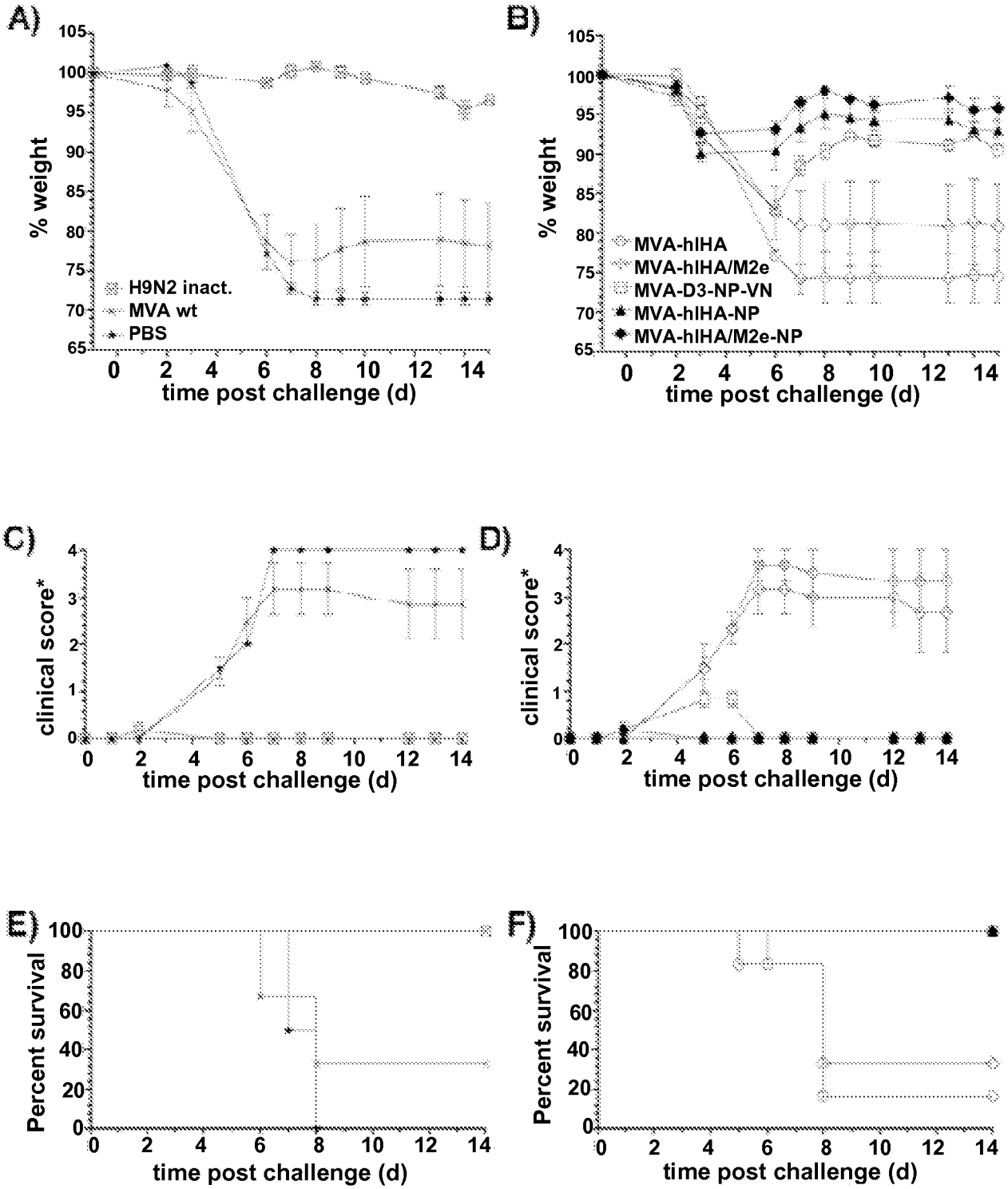
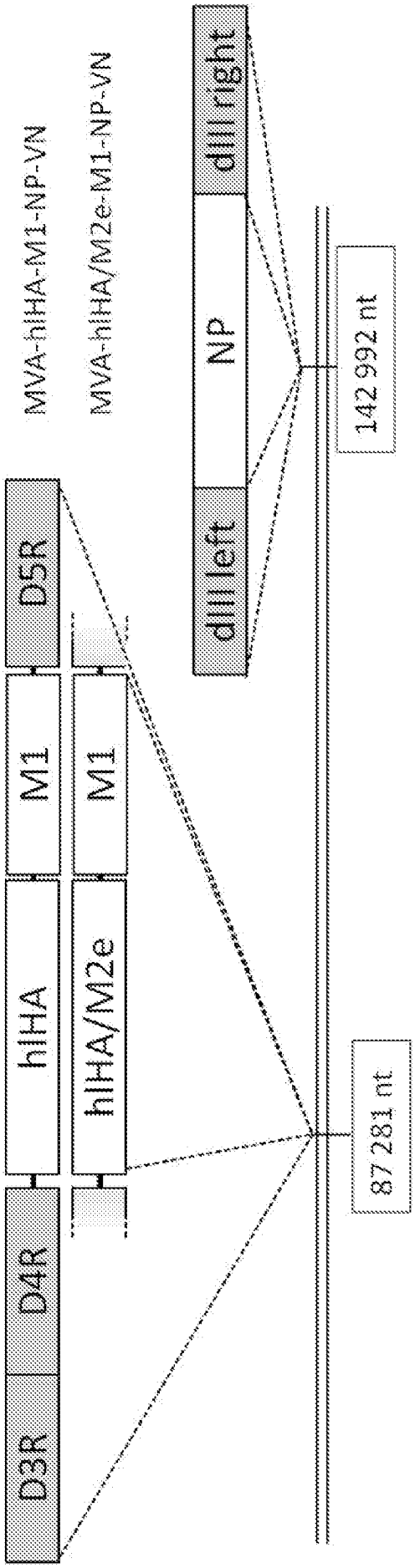

A)
% weight
time post challenge (d)
H9N2 inact.
MVA wt
PBS
B)
% weight
time post challenge (d)
MVA-hIHA
MVA-hIHA/M2e
MVA-D3-NP-VN
MVA-hIHA-NP
MVA-hIHA/M2e-NP
C)
clinical score*
time post challenge (d)
D)
clinical score*
time post challenge (d)
E)
Percent survival
time post challenge (d)
F)
Percent survival
time post challenge (d)

FIGURE 11


MVA-hIHA-M1-NP-VN
MVA-hIHA/M2e-M1-NP-VN
D3R
D4R
hIHA
M1
D5R
hIHA/M2e
M1
dIII left
NP
dIII right
87 281 nt
142 992 nt