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(54) Title: HSV-2-DELTA-gD VACCINES AND METHODS FOR THEIR PRODUCTION AND USE

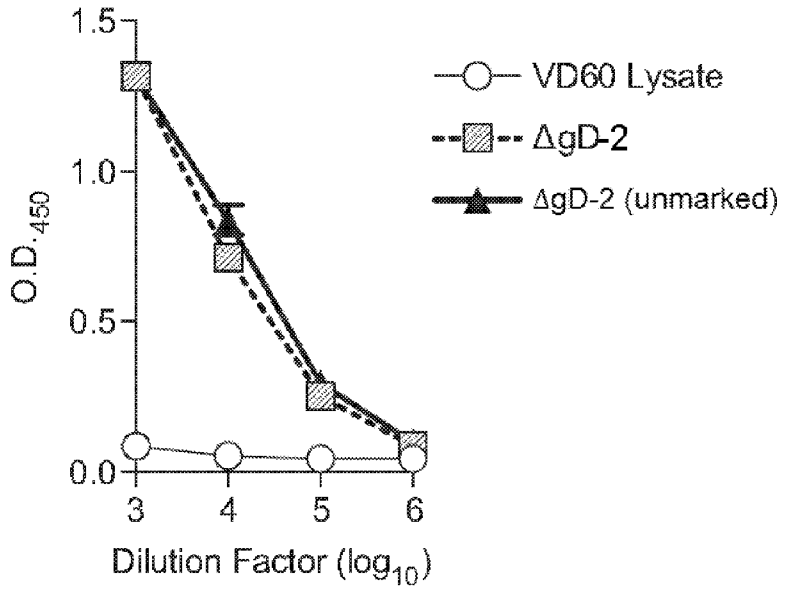

FIG. 2

(57) Abstract: Recombinant herpes simplex virus 2 (HSV-2) vaccine vectors, compositions and vaccines comprising such, and methods of use thereof are each provided.

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[Continued on next page]

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HSV-2-DELTA-gD VACCINES AND METHODS FOR THEIR PRODUCTION AND USE

STATEMENT OF GOVERNMENT SUPPORT

[0001] This invention was made with government support under grant numbers AI117321 and AI007501 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

[0002] Pathogen infections, including influenza and HIV viral infections, have a massive impact on global health. Vaccines have been developed for many pathogens, including both influenza and HIV viral infections, however they have failings and limitations. Thus, a novel vaccine strategy must be engineered and evaluated.

SUMMARY OF THE INVENTION

[0003] Disclosed herein are new and improved HSV-2-based vaccines for various antigenic targets, including influenza and HIV.

[0004] A process is provided for producing a vaccine vector directed against a heterologous antigen, the process comprising:

a) providing an HSV-2 genome comprising:

(i) a fully or partially deleted in a gene encoding HSV-2 glycoprotein D, and

(ii) a nucleic acid comprising a promoter-FP construct, wherein FP is a nucleic acid encoding a fluorescent protein;

b) co-transfecting a host cell with (i) the HSV-2 genome of a) and (ii) a linear DNA fragment encoding the heterologous antigen under conditions whereby allelic recombination occurs between the HSV-2 genome and the DNA fragment;

c) screening plaques resulting from b) to identify plaques not showing fluorescence under excitation light which elicits fluorescent protein fluorescence;

d) recovering from those plaques not showing fluorescence in c) recombinant HSV-2 viruses or virions so as to obtain a vaccine vector directed against the heterologous antigen.

[0005] Also provided is a recombinant herpes simplex virus-2 (HSV-2) having a genome encoding a heterologous antigen made by the process described herein.

[0006] Also provided is a recombinant herpes simplex virus-2 (HSV-2) having (i) a complete deletion of an HSV-2 glycoprotein D-encoding gene (referred to as ΔgD-2) in the genome thereof and (ii) (a) encoding: a promoter, a heterologous antigen signal sequence, a heterologous antigen or (b) encoding: a promoter, a heterologous antigen.

[0007] Also provided is a recombinant herpes simplex virus-2 (HSV-2) having (i) a partial deletion of an HSV-2 glycoprotein D-encoding gene in the genome thereof and (ii) (a) encoding, in order, an HSV-2 gD signal sequence, a heterologous antigen, an HSV-2 gD transmembrane domain, optionally an HSV-2 gD cytosolic domain, but not encoding an HSV-2 gD extracellular domain, or (b) encoding, in order, an HSV-2 gD signal sequence, a heterologous antigen, and transmembrane cytoplasmic tail of HSV-2 gD.

[0008] Also provided is an isolated cell comprising therein a recombinant HSV-2 virus as described herein, wherein the cell is not present in a human being.

[0009] Also provided is a vaccine composition comprising the recombinant HSV-2 virus as described herein.

[0010] Also provided is a pharmaceutical composition comprising a recombinant HSV-2 virus as described herein, and a pharmaceutically acceptable carrier.

[0011] Also provided is a method of eliciting and/or enhancing an immune response in a subject comprising administering to the subject an amount of (i) a recombinant HSV-2 virus as described herein; (ii) a vaccine as described herein; or (iii) a pharmaceutical composition as described herein, in an amount effective to elicit and/or enhance an immune response in a subject.

[0012] Also provided is a method of treating or reducing the likelihood of an influenza infection in a subject comprising administering to the subject an amount of (i) a recombinant HSV-2 virus as described herein; (ii) a vaccine as described herein; or (iii) a pharmaceutical composition as described herein, in an amount effective to treat or reduce the likelihood of an influenza infection in a subject.

[0013] Also provided is a method of treating or reducing the likelihood of an HIV infection in a subject comprising administering to the subject an amount of (i) a recombinant HSV-2 virus as described herein; (ii) a vaccine as described herein; or (iii) a pharmaceutical composition as described herein, in an amount effective to treat or reduce the likelihood of an HIV infection in a subject.

[0014] Also provided is a method of vaccinating a subject for influenza infection comprising administering to the subject an amount of (i) a recombinant HSV-2 virus as

described herein; (ii) a vaccine as described herein; or (iii) a pharmaceutical composition as described herein, in an amount effective to vaccinate a subject for influenza infection.

[0015] Also provided is a method of vaccinating a subject for HIV infection comprising administering to the subject an amount of (i) a recombinant HSV-2 virus as described herein; (ii) a vaccine as described herein; or (iii) a pharmaceutical composition as described herein, in an amount effective to vaccinate a subject for HIV infection.

[0016] Also provided is a method of quantitating a rate or amount of antibody-dependent cell-mediated killing (ADCK) in a population of cells comprising infecting a plurality of cells of the population of cells with a fluorescent protein-expressing recombinant HSV-2 that comprises a genome deleted for the gene encoding HSV-2 gD, under conditions permitting expression of the fluorescent protein in the cells, contacting the plurality of infected cells with an antibody-containing solution and a population of immune cells, and quantitating at one or more time points the amount of cells exhibiting fluorescent protein fluorescence and, optionally, one or more markers, so as to quantitate over time the amount of live infected cells, so as to thereby quantitating the rate or amount of ADCK in the population of cells.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] FIGS. 1A-1B. HSV-2 ΔgD::HA and HSV-2 ΔgD::HAstalk were created by co-transfection of plasmid DNA and ΔgD-2::$P_{EF1\alpha}$-RFP (B3x2.8, also referred to as ΔgD-2::RFP) genomic DNA and verified with PCR. FIG. 1A Purified pYUB2169 containing the desired gD::HA gene was cut with PacI and co-transfected into VD60 cells alongside HSV-2 ΔgD::PEF1α-RFP genomic DNA. Electroporation was used. Resultant RFP- plaques were purified three times and assessed for the presence of HA. FIG. 1B Recombinant viruses were verified by PCR amplification of the extracellular HA domain from plasmid and recombinant viral DNA. Primers used were located 100 base pairs (bp) upstream and downstream of the extracellular HA domain. Recombinant viruses both showed the desired band, as visualized by comparison to plasmid DNA. No band at that size was present in wells which contained DNA from ΔgD*2 (B^3x2.9)(Lanes 1 and 4). Lane 2 shows DNA from pYUB2169 containing full-length HA construct. Lane 3 shows DNA from recombinant HSV-2 ΔgD-2::HA (B^3x2.10) containing the non-codon optimized full-length extracellular domain of HA. Lane 5 shows DNA from recombinant HSV-2 ΔgD-2::HAstalk containing the codon-optimized stalk domain of HA. Lane 6 shows DNA from B^3x2.11.

[0018] FIG. 2 shows antibody response to vaccination with ΔgD-2::GFP (B^3x 2.7) or ΔgD-2 (B^3x 2.9).

[0019] FIG. 3 shows results of antibody-dependent cellular cytotoxicity of cells infected with ΔgD-2::GFP virus vs. unmarked ΔgD-2 virus.

[0020] FIG. 4. T-cell response to ΔgD-2::GFP virus vs. unmarked virus.

[0021] FIG. 5. ΔgD-2 vaccination protects against challenge with wild-type HSV-2 strain 4674, similar to protection with ΔgD-2::GFP.

[0022] FIG. 6. Construction of chimeric ΔgD-2::HA viruses and immune response in mice. The regions corresponding to the HA1 Stalk region, the HA1 Head region, the HA2 Stalk region, the HA2 Transmembrane region, and the HA2 Cytosolic region are indicated in the amino acid sequence and functional domains of A/Puerto Rico/1934/8 (PR8) HA (SEQ ID NO: 2). For chimeric ΔgD-2::HA viruses, the HA1 stalk, HA1 head (or 4 glycine residues), and HA2 stalk were fused to the transmembrane, cytosolic, and signal sequence domains of HSV-2 (G) gD. For the headless ΔgD-2::HA genes, the HA1 head domain was replaced with 4 glycine residues.

[0023] FIG. 7. Presence of chimeric ΔgD-2::HA in recombinant viruses verified by PCR. The lanes as follows: Lane 1: ΔgD-2genomic DNA, Lane 2: ΔgD-2::FL HA nOP genomic DNA, Lane 3: pBJJ1 plasmid DNA, Lane 4: ΔgD-2genomic DNA, Lane 5: ΔgD-2::HL HA nOP genomic DNA, Lane 6: pBJJ2 plasmid DNA. Primers used for PCR amplification were located just upstream and downstream of the HA extracellular domains. No exogenous promoter was inserted into the expression cassette. As a result, chimeric gene expression was regulated by the endogenous promoter.

[0024] FIG. 8. Mice vaccinated with ΔgD-2::FL HA nOP (β3χ1) and gD-2::HL HA OPT (β3χ3) are fully protected against HSV-2 challenge but do not form anti-HA IgGs. Mice were prime-boost vaccinated on days 0 and 21 with a control VD60 cell lysate or $1x10^6$ PFU of gD-2::FL HA nOP, gD-2::HL HA OPT, or ΔgD-2::RFP. FIG. 8A. At day 42, mice were challenged with a 10x LD_{90} of wild type HSV-2 4674. Mice vaccinated with gD-2::FL HA nOP and gD-2::HL HA OPT were fully protected from challenge. FIG. 8B. At day 40 post-prime vaccination, mice were bled to look at serum antibodies. ELISAs performed against soluble PR8 HA show the absence of HA-specific IgGs in mice.

[0025] FIG. 9 is a map of pEGFP-N1 expression plasmid. The PCMV::HA::SV40 polyA cassettes were restriction cloned into pBRL812, a plasmid containing >5.5 kB of the HSV-2 (G) genome upstream and downstream of US6. pBRL812 was cut with AsiSI and

PacI and co-transfected with ΔgD-2::RFP genomic DNA into VD60 cells. Allelic exchange was identified by lack of RFP expression and RFP negative plaques were picked and purified three times before verification of recombination by PCR.

[0026] FIG. 10. Map of the pBRL812 HSV recombination plasmid prepared as described in the above description of FIG. 9.

[0027] FIG. 11A. Verification of ΔgD-2::P_{CMV}-FL HA nOP. PCR using primers just upstream and downstream of the P_{CMV}::HA::SV40 PolyA cassette. In FIG. 11A, unlabeled lane: DNA ladder, Lane 1: pJHA1, Lane 2: 1.2.1 potential recombinant isolate, and Lane 3: negative control ΔgD-2 genomic DNA.

[0028] FIG. 11B. Verification of ΔgD-2::P_{CMV}-HL HA nOP using PCR using primers just upstream and downstream of the P_{CMV}::HA::SV40 PolyA cassette. Unlabeled lane: DNA ladder, Lane 1: pJHA2, lane 2: 2.2.2 potential recombinant isolate, Lane 3: 2.3.1 potential recombinant isolate, and Lane 4: negative control ΔgD-2 genomic DNA.

[0029] FIG. 11C. Verification of ΔgD-2::P_{CMV}-HL HA OPT. PCR using primers just upstream and downstream of the P_{CMV}::HA::SV40 PolyA cassette. Lane 1: pJHA4, Lane 2: 1.2.2 potential recombinant isolate, Lane 3: 1.2.3 potential recombinant isolate, unlabeled lane: DNA ladder. The latter (1.2.3 potential recombinant) isolate was expanded to make the original ΔgD-2::P_{CMV}-HL HA OPT stock.

[0030] FIG. 12: ΔgD-2::P_{CMV}-HA recombinant viruses all express HA. VD60 cells were infected with 3 MOI of ΔgD-2::P_{CMV}-FL HA nOP, ΔgD-2::P_{CMV}-HL HA nOP, or ΔgD-2::P_{CMV}-HL HA OPT. At 16 hours post-infection, cells were harvested and stained for HSV-protein and HA expression. HSV protein expression was measured using serum from mice vaccinated with ΔgD-2::RFP. HA expression was measured using monoclonal anti-HA stalk IgG C179. Cells were stained with either cell permeable or cell impermeable methods. Statistics were calculated by student's t test of geometric means between control fluorescence and fluorescence in wells stained for HA expression. Only ΔgD-2::P_{CMV}-FL HA nOP induced high levels of HA expression in infected cell membranes, but all three viruses induced expression as measured by cell-permeable staining.

[0031] FIG. 13 shows that ΔgD-2::P_{CMV}-FL HA nOP does not spread from cell to cell.

[0032] FIGS. 14A-14E. Kinetics of fluorescent protein expression and infection with HSV-2 ΔgD-2::RFP (ΔgD-2::RFP) compared to the parental HSV-2 ΔgD-2::GFP (ΔgD-2) strain. FIG. 14A. HSV-2 ΔgD-2::RFP (ΔgD-2::RFP) was created by transfecting VD60 cells simultaneously with HSV-2 ΔgD-2::GFP (ΔgD-2) genomic DNA and a plasmid

containing pEF1α::RFP flanked on either side by regions homologous to the HSV-2 (G) genome. Homologous recombination between genomic and plasmid DNA yielded ΔgD-2::RFP. Recombinant viruses were isolated by three rounds of plaque purification and verified with PCR and viral genome sequencing. FIGS. 14B and 14C. Vero cells were infected with 1 MOI of HSV-2 ΔgD-2 (B) or ΔgD-2::RFP. Kinetics of infection, RFP expression, and GFP expression were monitored over time. Both viruses show similar kinetics of infection. ΔgD-2 induced very little GFP expression even at 24 hours post-infection while ΔgD-2::RFP induced high levels of RFP expression beginning at 4.5 hours post-infection. FIGS. 14D and 14E. Images show Vero (D) and VD60 (E) cells at 12 hours post-infection with 1 MOI of ΔgD-2::RFP. Infected VD60 cells are forming syncytia, indicative of productive infection. Vero cells have not formed any syncytia. Images taken with deconvolution at 10x magnification.

[0033] FIGS. 15A-15C. Validation of novel rapid fluorometric antibody-dependent cell-mediated killing (RFADCK) assay in both Raw 264.7 and J774.1 cells. FIG. 15A. Highly expressing target cells were isolated by flow analysis using dual expression of membrane and live/dead markers. The proportion of cells expressing high levels of HSV proteins was then gated by determining the mean RFP intensity for populations of infected target cells. The percent difference in the proportion of this population between treated and untreated groups was then calculated as ADCK. FIG. 15B. Infected target cells were incubated in the presence or absence of serum from ΔgD-2::RFP prior to co-culture with J774.1 macrophages. Cells were fixed after 12 hours of co-culture and the proportions of RFP^{high} and RFP^{mid} target cells were quantified. In assay wells containing serum from ΔgD-2:RFP vaccinated mice, there were significantly fewer RFP^{high} cells at the end of the co-culture ($p<0.05$, mean = 70.5, 49.3), but not RFP^{mid} or $RFP^{high+mid}$ cells. Values represent 3 independent experiments done at least in duplicate. FIG. 15C. ADCK assays were performed in parallel using Raw 264.7 and J774.1 macrophages as effector cells. The number of RFP^{high} cells present in co-cultures containing serum from mice vaccinated with ΔgD-2:RFP or VD60 cell lysate were compared to that of co-cultures without serum. Serum from ΔgD-2:RFP vaccinated mice induced more killing in co-cultures containing both Raw 264.7 and J774.1 cells ($p=0.09$, $p<0.001$; mean = 18.8%, 38.3%). Data represents three independent experiments done in triplicate with J774.1 and Raw 264.7 cells at effector to target cell ratios of 10:1. Target cells used in all experiments were HEK 293. Statistics in B

were done using student's *t* tests. Statistics in C were done using one-way ANOVA. Error bars reflect SEM. * $p < 0.05$; *** $p < 0.001$.

[0034] FIG. 16. ADCC and ADCP are not easily distinguishable but are mediated by Raw 264.7 macrophages in the presence of serum from HSV-2 ΔgD-2::RFP vaccinated mice. Quantification of the number of infected cells that died during three movies suggests that the addition of serum from HSV-2 ΔgD-2::RFP vaccinated mice induces more killing than serum from mice given a control VD60 cell lysate when compared to movies taken of co-cultures lacking of serum (p=0.14, mean=18.5, 3.7, n=58 infected cells for both groups). Images represent a partial frame of one movie taken at 60x magnification. Images were taken every 15 minutes for 24 hours. HEK 293 cells were used as target cells. Raw 264.7 cells were used as macrophages. All assays were done at a 10:1 ratio of effector to target cells. Statistics were done using a student's *t* test.

[0035] FIGS. 17A-17B. The ADCK assay can be adapted to knockout mouse strains and other model organisms. FIG. 17A. ADCK assay was carried out using bone marrow-derived macrophages (BMDMs) from both $Fc\gamma R^{-/-}$ and WT mice. Baseline killing of infected target cells was determined in co-cultures containing serum from VD60 lysate vaccinated mice. Data represents the percent difference between co-cultures containing serum from ΔgD-2::RFP vaccinated mice and the baseline. At an embodiment effector:target ratio of 10:1, WT BMDMs carried out significantly more ADCK in the presence of HSV-2 ΔgD-2::RFP vaccinated serum than $Fc\gamma R^{-/-}$ BMDMs (p<0.05). Killing of infected cells by $Fc\gamma R^{-/-}$ BMDMs in co-cultures containing serum from ΔgD-2::RFP vaccinated mice was indistinguishable from that of co-cultures containing serum from VD60 lysate mock-vaccinated mice. FIG. 17B. ADCK assay was carried out using BMDMs derived from naïve guinea pig marrow. Baseline ADCK was determined by co-cultures containing serum from naïve animals. An effector:target ratio of 5:1 was used due to limited marrow availability. At this ratio, BMDMs in co-cultures containing pooled serum from ΔgD-2 vaccinated guinea pigs carried out significantly more ADCK than BMDMs in co-cultures containing pooled serum from VD60 mock-vaccinated guinea pigs (p<0.001). Each pooled sample contained serum from 6-9 animals. Data represents two independent assays run in triplicate. HEK 293 cells were used as target cells. Statistics were done by student's *t* test. Error bars reflect SEM. * $p < 0.05$; *** $p < 0.001$.

[0036] FIG. 18: Anti-HA antibodies elicited by vaccination with various PR8 influenza A virus (IAV) hemagglutinin (HA) HSV-2 recombinants (see Legend in figure).

[0037] FIG. 19: Anti-HA antibody isotype elicited by vaccination with the HSV-2 recombinant ΔgD-2::FL HA nOP. ** $p < 0.01$.

[0038] FIG. 20: Anti-PR8 IgG Isotype ELISA.

[0039] FIGS. 21A-21C: Mice vaccinated with ΔgD-2::*FL* HA_{PR8} (ΔgD-2::HA_{PR8}) are fully protected from challenge with PR8. Mice were prime-boost vaccinated subcutaneously 3 weeks apart with $5x10^6$ PFU of ΔgD-2::RFP or ΔgD-2::HA_{PR8} or mock vaccinated with VD60 cell lysate. FIG. 21A. The mice were bled one-week post-boost and serum neutralization titer was measured against A/Puerto Rico/1934/8 IAV (PR8). Mice immunized with ΔgD-2::HA_{PR8} developed significant neutralizing Ab titers against PR8 (mean = 304). Dotted line represents the limit of detection for the assay. FIGS. 21B and 21C. Three weeks post-boost, mice were challenged intranasally with a $6xLD_{50}$ of PR8. Mice were sacrificed when they reached 75% of their initial weight. Mice immunized with ΔgD-2::HA_{PR8} were fully protected from PR8 challenge while mice that received control vaccinations all succumbed to infection before day 9. Statistics for neutralization titer were calculated by ANOVA. Survival statistics were calculated by Mantel-Cox log-rank test *$p<0.05$; **$p<0.01$; ***$p<0.001$.

[0040] FIGS. 22A-22C show that recombinant gD-2::HA_{PR8} expresses high levels of PR8 protein. In FIG. 22A, ΔgD-2::RFP DNA was co-transfected into VD60 cells alongside an HA expression cassette containing the hemagglutinin (HA) gene from IAV H1N1 strain A/Puerto Rico/1934/8 (PR8) downstream of P_{CMV} and upstream of a poly-adenylation signal. In FIG. 22B, extracellular and intracellular HA expression was measured by flow cytometry in Vero and VD60 cells infected with 3 MOI of ΔgD-2, ΔgD-2::HA_{PR8}, or ΔgD-2 containing a truncated version of the PR8 HA expression cassette (ΔgD-2::HL HA_{PR8}).

[0041] FIGS. 23A-23F show that mice immunized with ΔgD-2::HA_{PR8} develop high titers of functional and isotype switched anti-PR8 antibodies. Mice were prime-boost vaccinated 21 days apart with either VD60 cell lysate, ΔgD-2::RFP vector, or ΔgD-2::HA_{PR8}. At day 28 post-prime, serum was collected for analysis. FIGS. A-E. Anti-PR8 antibodies were measured by ELISA against purified HA PR8 protein. Mice immunized with ΔgD::HA_{PR8} developed isotype switch anti-PR8 HA antibodies that were predominantly IgG2c and IgG2b. In FIG. 23F, serum from mice immunized with ΔgD-2::HA_{PR8} induced significant hemagglutination inhibition compared to serum from mice immunized with ΔgD-2 ($p<0.001$; $mean_{HAI\ titer} = 80$). Mice immunized with ΔgD-2::RFP did not develop any hemagglutination inhibiting antibodies. All graphs show 1

representative experiment with n=5 mice/group. Statistics were calculated using Student's T-test. ** $p < 0.01$.

[0042] FIGS. 24A-24L show that mice immunized with ΔgD-2::HA_{PR8} develop protection against IAV challenge. Mice were prime-boost immunized 21 days apart with VD60 cell lysate, ΔgD-2, or ΔgD-2*::HA_{PR8}*, bled at day 28 post-prime, and challenged intranasally 14 days later with a 6x LD_{50} of IAV. FIGS. 24A-24C, mice immunized with ΔgD-2::HA_{PR8} developed significant neutralizing titers to PR8 (mean $titer_{PR8}$ = 1:304; p_{VD60} < 0.01; $p_{\Delta gD-2}$ < 0.05; n=10 mice/group). Mice immunized with ΔgD-2::HA_{PR8} were also fully protected from weight loss and mortality followed by challenge with PR8 ($p < 0.001$, n=5 mice/group). FIGS. 24D-24F. Mice immunized with ΔgD-2*::*HA_{PR8} developed significant neutralizing antibodies against A/California/2009 H1N1 (A/Cal/2009) IAV (mean $titer_{A/Cal}$ = 26; $p_{VD60} < 0.01$ $p_{\Delta gD-2} = 0.1$; n=10 mice/group). Mice immunized with ΔgD*::*HA_{PR8} were partially protected from weight loss and mortality following challenge with A/Cal/2009 (p <0.05, n=15 mice/group). As shown in FIGS. 24G-24I, mice immunized with ΔgD-2*::*HA_{PR8}, ΔgD-2*::RFP*, or VD60 cell lysate did not develop neutralizing antibody titers against influenza strains A/Victoria/3/75 H3N2 (A/Vic), and A/Aichi/68 H3N2 (X-31) and were not protected from weight loss and mortality following challenge ($n_{neutralization}$ = 10 mice/group; $n_{A/Vic\ challenge}$ = 15 mice/group; $n_{X-31\ challenge}$ = 5 mice/group). Neutralization titers were measured using microneutralization assays against the respective strains. Mice were sacrificed after reaching 70% of starting weight. Statistics for neutralization assays done using 3-way ANOVA tests. Statistics for survival done using Mantel-Cox log-rank tests. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

[0043] FIGS. 25A-25F. Mice immunized with ΔgD-2::HA_{PR8} develop fully protective ADCC immunity against HSV-2. Mice were prime-boost vaccinated 21 days apart with either VD60 cell lysate, ΔgD-2, or ΔgD-2::HA_{PR8}. In FIGS. 25A and 25B, the mice were bled 1-week post-boost and the serum was analyzed by ELISA. Mice that received ΔgD-2 or ΔgD-2::HA_{PR8} generated similarly high levels of HSV-specific IgG (FIG. 25A). Additionally, these IgGs were predominantly IgG2c. In FIG. 25C, rapid fluorometric antibody-dependent cell-mediated killing (RFADCK) assay was carried out using the same serum as in FIGS. 25A and 25B. Sera from mice vaccinated with ΔgD-2 or ΔgD-2::HA_{PR8} elicited significant ADCK activity in the presence of J774.1 macrophages and ΔgD-2 infected cells compared with serum from mice given VD60 cell lysate ($p < 0.01$). There was no difference between the ADCK activity elicited by sera from mice vaccinated with ΔgD-2

and ΔgD-2::HA_{PR8}. In FIGS. 25D to 25F, at 21 days post-boost, mice were challenged by skin scarification with a 10x LD_{90} of HSV-2 4674. Mice that received VD60 cell lysate succumbed to HSV-2 by 10 and developed significant epithelial and neurological disease. Mice that received ΔgD-2 and ΔgD-2::HA_{PR8} were fully protected from morbidity and mortality following challenge. n=5 mice/group from a representative experiment. Statistics for RFADCK assay done using 3-way ANOVA tests. Statistics for survival done using Mantel-Cox log-rank tests. ** $p < 0.01$; **** $p < 0.0001$.

[0044] FIG. 26 shows the results of mFcγRIV ADCC reporter bioassay response to serially diluted serum samples collected from mice vaccinated with HSV-2 ΔgD::HA, HSV-2 ΔgD::RFP and inactivated A/Puerto Rico/8/1934 H1N1 virus (PR8). Madin-Darby Canine Kidney (MDCK) cells infected with PR8 virus were used as target cells and mFcγRIV expressing Jurkat T cells were used as effector cells. The target cells were incubated with serially diluted serum samples and effector cells. Bio-Glo™ Reagent was added, and luminescence was measured. The recombinant HSV-2 ΔgD-2::HA vaccinated mice showed significantly higher activation of mFcγRIV receptors in comparison to mice vaccinated with HSV-2 ΔgD-2::RFP and inactivated PR8 virus. The p value for significance is <0.05. Symbols: **$P \leq 0.01$, ***$P \leq 0.001$, **** $P \leq 0.0001$.

[0045] FIG. 27 is a map of the plasmid ZM109F.PB4, which was used to amplify full length HIV-1 *Env (Clade C)* and *Rev*, together with partial *Nef* of HIV-1.

[0046] FIG. 28 is a map of the pBkk412 plasmid.

[0047] FIG. 29A. Screen of transformed *E.coli* colonies for the presence of the cloned insert.

[0048] FIG. 29B. Analysis by PCR to identify clones having the correct nucleic acid size.

DETAILED DESCRIPTION

[0049] A process is provided for producing a vaccine vector directed against a heterologous antigen, the process comprising:

a) providing an HSV-2 genome comprising:

(i) a fully or partially deleted in a gene encoding HSV-2 glycoprotein D, and

(ii) a nucleic acid comprising a promoter-FP construct, wherein FP is a nucleic acid encoding a fluorescent protein;

b) co-transfecting a host cell with (i) the HSV-2 genome of a) and (ii) a linear DNA fragment encoding the heterologous antigen under conditions whereby allelic recombination occurs between the HSV-2 genome and the DNA fragment;

c) screening plaques resulting from b) to identify plaques not showing fluorescence under excitation light which elicits fluorescent protein fluorescence;

d) recovering from those plaques not showing fluorescence in c) recombinant HSV-2 viruses or virions so as to obtain a vaccine vector directed against the heterologous antigen.

[0050] In embodiments, the promoter of the promoter-FP construct is a heterologous promoter.

[0051] A process is also provided for producing a vaccine vector directed against an antigen, the process comprising:

a) providing an HSV-2 genome comprising:

(i) a deletion in a gene encoding HSV-2 glycoprotein D, and

(ii) a nucleic acid comprising an $P_{EF1\alpha}$-RFP construct, where $P_{EF1\alpha}$ is a promoter of Elongation Factor 1α gene and RFP is a nucleic acid encoding red fluorescent protein, wherein $P_{EF1\alpha}$ and RFP are fused together ($P_{EF1\alpha}$-RFP);

b) co-transfecting a host cell with (i) the HSV-2 genome of a) and (ii) linear DNA fragments encoding, in order, (i) HSV-2 gD signal sequence, the antigen, HSV-2 gD transmembrane domain, HSV-2 gD cytosolic domain, but not encoding a HSV-2 gD extracellular domain, or (ii) HSV-2 gD signal sequence, the antigen, and transmembrane cytoplasmic tail of HSV-2 gD, under conditions permitting allelic recombination;

c) screening plaques resulting from b) to identify plaques not showing red fluorescence under excitation light which elicits red fluorescent protein fluorescence;

d) recovering from those plaques not showing red fluorescence in c) recombinant HSV-2 viruses or virions so as to obtain a vaccine vector directed against the antigen.

[0052] A variety of promoters can be employed. Heterologous promoters are preferred including those with high efficiency. Such promoters include the CMV promoter, including the cytomegalovirus major immediate-early promoter. The human elongation factor-1 alpha (EF-1 alpha) constitutive promoter is of human origin and can be used to drive ectopic gene expression *in vitro* and *in vivo*. A variety of PEF-1 alpha may be used. The human EF1α gene sequence is known in the art, for example see NCBI accession No. J04617. Other heterologous promoters known in the art and usable in the invention include, but are not limited to, CMV enhancer fused to the chicken beta-actin promoter (CAG), mouse

cytomegalovirus (mouse CMV), Chinese hamster elongation factor-1α (CHEF-1α), and phosphoglycerate kinase (PGK).

[0053] In embodiments of the processes, the host cell is a complementing cell, for example a cell that phenotypically complements for HSV-1 glycoprotein D. In embodiments of the processes, the host cell is a VD60 cell, which phenotypically complements HSV-1 gD. In embodiments, the vaccine vector produced is genotypically deleted for HSV-2 gD and is phenotypically complemented for HSV-1 gD on a lipid bilayer thereof. In embodiments, the vaccine vector produced does not genotypically encode any HSV gD.

[0054] In embodiments, the host cell is co-transfected with (i) the HSV-2 genome of a) and (ii) a linear DNA fragment encoding, in order, (i) HSV-2 gD signal sequence, the heterologous antigen, HSV-2 gD transmembrane domain, HSV-2 gD cytosolic domain, but not encoding a HSV-2 gD extracellular domain, or (ii) HSV-2 gD signal sequence, the heterologous antigen, and transmembrane cytoplasmic tail of HSV-2 gD.

[0055] In embodiments, the host cell is co-transfected with (i) the HSV-2 genome of a) and (ii) linear DNA fragments encoding, in order, (i) a promoter, the heterologous antigen, and optionally a poly-A signal.

[0056] In embodiments, the co-transfecting is effected by electroporation.

[0057] Examples of nucleic acid-encodable fluorescent proteins for use in the invention include red, far red, yellow, green, orange, cyan or photo switchable fluorescent protein. Examples of such proteins are well known in the art. Supplies include Molecular Probes (ThermoFisher USA) and Takara (USA). In an embodiment, the fluorescent protein is Red Fluorescent Protein. Fluorescent proteins with an excitation range of 554-584 nanometers (nm) and an emission range of 562-610 nm are preferred. Examples include Red Fluorescent Protein, mCherry, mTomato, J-Red and mOrange. RFP has an excitation of 556 nm and emission of 584 nm. Alternatively, firefly luciferase or nano-luciferase can be used.

[0058] In embodiments, the antigen is not an HSV-2 antigen, i.e. it is a heterologous antigen. As used herein, an antigen is heterologous when it is heterologous relative to HSV-2, i.e. is not naturally found on or in a wildtype HSV-2.

[0059] The heterologous antigen can be derived from a living organism, comprising for example, a virus, a bacteria, a parasite, a human cell, an animal cell, or a combination thereof. The heterologous antigen can be a surface protein or a non-surface protein.

[0060] The virus can be a pathogenic virus, examples of which include cytomegalovirus (CMV), coxsackie virus, Crimean-Congo hemorrhagic fever virus, chikungunya virus, dengue virus, Dhori virus, Eastern equine encephalitis (EEE) virus, ebola virus, Epstein Barr virus (EBV), hepatitis virus, herpesvirus, human immunodeficiency (HIV) virus, human papilloma virus, human SARS corona virus, human T lymphotropic virus (HTLV), influenza virus, measles virus, mumps virus, Norwalk virus, rabies virus, rotavirus, rubella virus, severe fever with thrombocytopenia syndrome (SFTS) virus, respiratory syncytial virus (RSV), varicella zoster virus, Western equine encephalitis virus, West Nile virus, yellow fever virus, Zika virus, or a combination thereof.

[0061] The bacteria can be a pathogenic bacteria, examples of which include *Bacillus sp., Baronella sp., Bordatella sp., Borelli asp., Brucella sp., Campylobacter sp., Chlamydia sp., Clostridium sp., Corynebacterium sp., Enterococcus sp., Escherichia sp., Haemophilis sp., Helicobacter sp., Legionella sp., Leptospira sp., Listeria sp., Mycobacterium sp., Mycoplasma sp., Neisseria sp., Rickettsia sp., Pseudomonas sp., Salmonella sp., Shigella sp., Staphylococcus sp., Streptococcus sp., Treponema sp., Vibrio sp., Yersinia sp.*, or a combination thereof.

[0062] The parasite can be a pathogenic parasite, examples of which include *Acanthamoeba spp., Balamuthia spp., Babesia sp., Balantidium coli, Blastocystic sp., Cryptospiridium sp., Cyclospora cayetanensis, Entamoeba histolytica, Giardia lamblia, Isospora bello, Leishmania sp., Naegleria foweri, Plasmodium sp., Rhinosporidium seeberi, Sarcocystis sp., Toxoplasma gondii, Trichomonas sp., Trypanosoma sp.*, or a combination thereof.

[0063] The human cell or animal cell can be, for example, a cancer cell.

[0064] In embodiments, the heterologous antigen is an influenza antigen. In embodiments, the heterologous antigen is an influenza hemagglutinin (HA) antigen. In embodiments, the HA antigen is a full-length HA extracellular domain or is a HA stalk domain.

[0065] In embodiments, the heterologous antigen is an HIV antigen. In embodiments, the HIV antigen is an Env gp145.

[0066] In embodiments, the heterologous antigen is under control of an upstream CMV promoter and has a downstream SV40 poly-A signal. The SV40 poly-A signal is known in the art. It promotes polyadenylation and transcription termination.

[0067] In embodiments, the promoter is a promoter of Elongation Factor 1α gene ($P_{EF1\alpha}$) and wherein $P_{EF1\alpha}$ and FP are fused together ($P_{EF1\alpha}$-FP).

[0068] In embodiments, the nucleic acid is codon-optimized for expression. See, for example, Table 4 in the examples hereinbelow.

[0069] Also provided is a vaccine vector made by the process described herein.

[0070] Also provided is a recombinant herpes simplex virus-2 (HSV-2) having a genome encoding a heterologous antigen made by the process described herein.

[0071] Also provided is a recombinant herpes simplex virus-2 (HSV-2) having (i) a complete deletion of an HSV-2 glycoprotein D-encoding gene in the genome thereof and (ii) (a) encoding: a promoter, a heterologous antigen signal sequence, and a heterologous antigen or (b) encoding: a promoter and a heterologous antigen.

[0072] Also provided is a recombinant herpes simplex virus-2 (HSV-2) having (i) a partial deletion of an HSV-2 glycoprotein D-encoding gene in the genome thereof and (ii) (a) encoding, in order, an HSV-2 gD signal sequence, a heterologous antigen, an HSV-2 gD transmembrane domain, optionally an HSV-2 gD cytosolic domain, but not encoding an HSV-2 gD extracellular domain, or (b) encoding, in order, an HSV-2 gD signal sequence, a heterologous antigen, cytosolic domain of HSV-2 gD.

[0073] Also provided is a recombinant herpes simplex virus-2 (HSV-2) having (i) a partial deletion of an HSV-2 glycoprotein D-encoding gene in the genome thereof and (ii) (a) encoding, in order, an HSV-2 gD signal sequence, a heterologous antigen, an HSV-2 gD transmembrane domain, an HSV-2 gD cytosolic domain, but not encoding an HSV-2 gD extracellular domain, or (b) encoding, in order, an HSV-2 gD signal sequence, a heterologous antigen, and transmembrane cytoplasmic tail of HSV-2 gD.

[0074] In embodiments, the recombinant HSV-2 further comprises a parasitic surface glycoprotein on a lipid bilayer thereof, wherein the parasite is a parasite of a mammal.

[0075] In an embodiment, the HSV-2 glycoprotein D-encoding gene is an HSV-2 *Us6* gene. (For example, see Dolan et al. *J Virol.* 1998 March; 72(3): 2010–2021. (PMCID: PMC109494) "The Genome Sequence of Herpes Simplex Virus Type 2" for HSV-2 genome and *Us6* gene, hereby incorporated by reference in its entirety). In embodiments, the HSV-2 glycoprotein D-encoding gene is equivalent of an HSV-2 *Us6* gene. Such equivalents are easily identifiable by those of skill in the art using readily available sequencing and alignment tools.

[0076] In embodiments, the heterologous antigen is an influenza antigen. In embodiments, the heterologous antigen is an influenza hemagglutinin (HA) antigen.

[0077] In embodiments, the HA antigen is a full-length HA extracellular domain or is a HA stalk. In embodiments, the full-length HA includes a HA signal sequence. In embodiments, the HA is an HA of a human influenza A or human influenza B. Examples of the hemagglutinin gene ("HA") include GenBank V01098.1; NCBI Reference Sequence: NP_040980.1. HA gene sequences, and the mature hemagglutinin peptide sequences, are well known in the art, and multiple HA sequences are available to those skilled in the art at the NCBI database. In addition, one skilled in the art can readily identify the commonly discussed stalk, extracellular domain and other regions of hemagglutinin. In addition, seasonal influenza virus strain sequences, including the HA sequence, are routinely sequenced and identified in the art.

[0078] Other influenza genes, as heterologous antigens, that can be added to ΔgD-2 by allelic exchange with B3x2.8 (ΔgD-2::$P_{EF1\alpha}$-RFP) include neuraminidase (NA), matrix protein 1 (M1), influenza A virus (IAV) matrix protein 2 (M2), influenza B virus (IBV) matrix protein 2 (M2), nucleoprotein (NP), and influenza B virus NB. A combination comprising at least one of the foregoing can also be used. The corresponding modified headless HA gene for each strain can be added as well as a version of each headless antigen where a trimerization domain has been added to increase stability. Table 1 provides non-limiting influenza examples of other such antigen genes. Other examples include inserting the HA gene from A/Vietnam/1203/04, the HA, headless HA, NA, M1, M2, NP, and NB genes from, e.g., B/Yamagata/16/1988 and B/Victoria/2/1987 strains.

Table 1 - Genbank Sequence Accession Codes for Examples of Exemplary Influenza Antigens

Antigens	A/Puerto Rico/1934/8 H1N1	A/Californi a/04/2009 Pandemic H1N1 swine flu	A/Hong Kong/156/ 97 Avian H5N1	A/New York/35/20 17 Seasonal H3N2	A/Hong Kong/213/ 2003 Avian H5N1	A/Shangh ai/01/2014 Avian H7N9	A/swine/M issouri/212 4514/2006 Swine H2N3	A/Italy/3/ 2013 Avian H7N7	A/Netherla nds/602/20 09 H1N1	A/Hong Kong/33982 /2009 H9N2
HA		KX136139	AF028709	CY256029	AB212054	KJ411975	EU258939	KF918337	CY148123	KF188316
NA	CY121111	KX136323	AF036357	CY256031	AB212056	KJ411977	EU258937	KF918339	CY039528	KF188318
M1	KC866600	KX135622	AF036358	CY256032	AB212057	KJ411976	EU258938	KF918340	CY046944	KF188319
M2	KC866600	KX135622	AF036358	CY256032		KJ411976	EU258938	KF918340	CY046944	KF188319
NP	CY148246.1	KX136793	AJ291400	CY256030	AB212055	KJ411978	EU258936	KF918338	CY046943	KF188317

[0079] One method for generating the recombinant HSV-2 gD -/- viral vector expressing HIV or Influenza A virus antigens is as follows:

1. Generating an engineered HSV-2 genome by partially deleting the glycoprotein D encoding gene Us6 such that only glycoprotein D signal sequence and the transmembrane-cytoplasmic domain sequence is left (no extracellular domain sequence present). The deleted portion of the glycoprotein D gene is replaced with a $P_{EF1\alpha}$-RFP gene wherein red fluorescent protein (RFP) is expressed under the control of a $P_{EF1\alpha}$ promoter.

2. A cosmid pYUB2169 can be engineered to express a gene for a heterologous antigen (e.g., HIV envelope protein antigen Env gp145 or Influenza virus HA stalk antigen) of choice that is flanked by sequences of the HSV-2 signal sequence and the transmembrane-cytoplasmic domain sequence on either respective side.

3. Both HSV-2 gD-/- RFP+ genome of (1) and engineered cosmid pYUB2169 of (2) can be introduced into a VD60 cell line.

4. Within the VD60 cell line, homologous recombination occurs between the HSV-2 partially deleted glycoprotein D gene (RFP gene inserted) of (1) and the pYUB2169-HIV Env gp145 or Influenza A virus HA stalk antigen expressing gene of (2). A successful recombination is expected to lead to loss of RFP gene and generation of a HSV-2 gD-/- virus expressing a heterologous HIV or Influenza virus antigen (see FIG. 1A-1B).

5. VD60 cell culture plaques that are negative for RFP expression contain HSV-2 gD-/- the virus particles are expected to express HIV Env gp145 or I influenza virus HA stalk antigen.

6. The HSV-2 gD-/- virus expressing a heterologous HIV Env gp145 or Influenza A virus HA stalk antigen thus generated can be used to vaccinate a person and elicit at strong antibody mediated response and protective immunity against HIV or Influenza Virus.

[0080] In embodiments, the heterologous antigen is an HIV antigen. In embodiments, the HIV antigen is an HIV-1 or HIV-2 antigen. For example, the heterologous antigen is an HIV-1 antigen. In embodiments, the HIV is a C-subtype. In embodiments, the HIV antigen is an Env, Pol, Gag, or Nef. In an embodiment, the HIV antigen is an Env antigen. In embodiments, the HIV antigen is a C-subtype Env antigen. In embodiments, the antigen is an Env gp145. In embodiments, the heterologous antigen is a fully intact membrane-proximal external region (MPER). In embodiments, the heterologous antigen is extended by a polylysine tail. In embodiments, the heterologous antigen is not extended by a polylysine tail. HIV gp145 Env protein sequences are readily identifiable by alignment tools, and are

routine to sequence. In embodiments, the ectodomain of HIV Env gp145 is fused with the signal peptide and transmembrane cytoplasmic tail of HSV gD.

[0081] Also provided is a cell comprising therein a recombinant virus as described herein, wherein the cell is not present in a human being.

[0082] Also provided is a vaccine composition comprising a recombinant virus as described herein. In embodiments, the vaccine comprises an adjuvant which is not derived from the HSV-2. Adjuvants are well known in the art and include alum, oil-in-water or water-in-oil emulsions, aluminum salts such as aluminum hydroxide, aluminum phosphate, and aluminum potassium sulfate, and monophosphoryl lipid A. In embodiments, the vaccine does not comprise an adjuvant.

[0083] Also provided is a pharmaceutical composition comprising a recombinant HSV-2 virus as described herein, and a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are well-known in the art.

[0084] Also provided is a method of eliciting and/or enhancing an immune response in a subject, the method comprising administering to the subject an amount of (i) a recombinant virus as described herein; (ii) a vaccine as described herein; or (iii) a pharmaceutical composition as described herein, in an amount effective to elicit and/or enhance immune response in a subject.

[0085] Also provided is a method of eliciting and/or enhancing an immune response in a subject against a pathogen expressing a heterologous antigen, the method comprising administering to the subject an amount of (i) a recombinant virus as described herein; (ii) a vaccine as described herein; or (iii) a pharmaceutical composition as described herein, in an amount effective to elicit and/or enhance immune response in a subject, wherein the recombinant virus of (i), (ii) or (iii) expresses the heterologous antigen.

[0086] Also provided is a method of treating or reducing the likelihood of an influenza infection in a subject, the method comprising administering to the subject an amount of (i) a recombinant virus as described herein; (ii) a vaccine as described herein; or (iii) a pharmaceutical composition as described herein, in an amount effective to treat or reduce the likelihood of an influenza infection in a subject. For treating or reducing likelihood of influenza, the heterologous antigen is an influenza HA antigen. Also provided is a method of vaccinating a subject for influenza infection comprising administering to the subject an amount of (i) a recombinant virus as described herein; (ii) a vaccine as described herein; or

(iii) a pharmaceutical composition as described herein, in an amount effective to vaccinate a subject for influenza infection.

[0087] Also provided is a method of treating or reducing the likelihood of an HIV infection in a subject, the method comprising administering to the subject an amount of (i) a recombinant virus as described herein; (ii) a vaccine as described herein; or (iii) a pharmaceutical composition as described herein, in an amount effective to treat or reduce the likelihood of an HIV infection in a subject. For treating HIV or reducing likelihood of HIV, the heterologous antigen is an HIV antigen. Also provided is a method of vaccinating a subject for HIV infection comprising administering to the subject an amount of (i) a recombinant virus as described herein; (ii) a vaccine as described herein; or (iii) a pharmaceutical composition as described herein, in an amount effective to vaccinate a subject for HIV infection.

[0088] Also provided is a method of eliciting and/or enhancing an immune response in a subject, the method comprising administering to the subject an amount of a recombinant herpes simplex virus-2 (HSV-2) made by a process described herein and comprising (i) a complete deletion of an HSV-2 glycoprotein D-encoding gene in the genome thereof and (ii) encoding a promoter, a influenza hemagglutinin (HA) antigen signal sequence, and an HA antigen in an amount effective to elicit and/or enhance an immune response in a subject.

[0089] Also provided is a method of treating or reducing the likelihood of an influenza infection in a subject, the method comprising administering to the subject an amount of a recombinant herpes simplex virus-2 (HSV-2) made by a process described herein and comprising (i) a complete deletion of an HSV-2 glycoprotein D-encoding gene in the genome thereof and (ii) encoding a promoter, a influenza hemagglutinin (HA) antigen signal sequence, and an HA antigen in an amount effective to treat or reduce the likelihood of an influenza infection in a subject.

[0090] Also provided is a method of vaccinating a subject for influenza infection, the method comprising administering to the subject an amount of a recombinant herpes simplex virus-2 made by a process described herein and comprising (i) a complete deletion of an HSV-2 glycoprotein D-encoding gene in the genome thereof and (ii) encoding a promoter, a influenza hemagglutinin (HA) antigen signal sequence, and an HA antigen in an amount effective to vaccinate a subject for influenza infection.

[0091] In embodiments, the HA antigen is a full-length HA extracellular domain.

[0092] In embodiments, the methods further comprise, subsequent to an initial administration of the recombinant herpes simplex virus-2 encoding a full-length HA extracellular domain, administering one or more amounts of a recombinant herpes simplex virus-2 having (i) a complete deletion of an HSV-2 glycoprotein D-encoding gene in the genome thereof and (ii) encoding a promoter, a HA antigen signal sequence, and an HA stalk, but not encoding a full-length HA.

[0093] In an embodiment of the methods or processes, the HSV-2 glycoprotein D comprises the amino acid sequence set forth in SEQ ID NO:1:

MGRLTSGVGTAALLVVAVGLRVVCAKYALADPSLKMADPNRFRGKNLPVLDQLT
DPPGVKRVYHIQPSLEDPFQPPSIPITVYYAVLERACRSVLLHAPSEAPQIVRGASDE
ARKHTYNLTIAWYRMGDNCAIPITVMEYTECPYNKSLGVCPIRTQPRWSYYDSFSA
VSEDNLGFLMHAPAFETAGTYLRLVKINDWTEITQFILEHRARASCKYALPLRIPPA
ACLTSKAYQQGVTVDSIGMLPRFIPENQRTVALYSLKIAGWHGPKPPYTSTLLPPEL
SDTTNATQPELVPEDPEDSALLEDPAGTVSSQIPPNWHIPSIQDVAPHHAPAAPSNPG
LIIGALAGSTLAVLVIGGIAFWVRRRAQMAPKRLRLPHIRDDDAPPSHQPLFY (HSV-2 reference strain HG52)

[0094] In an embodiment, the HSV-2 in which the HSV-2 glycoprotein D-encoding gene is deleted is an HSV-2 having a genome (prior to the deletion) as set forth in one of the following Genbank listed sequences: HSV-2(G) (KU310668), HSV-2(4674) (KU310667), B3x1.1 (KU310657), B3x1.2 (KU310658), B3x1.3 (KU310659), B3x1.4 (KU310660), B3x1.5 (KU310661), B3x2.1 (KU310662), B3x2.2 (KU310663), B3x2.3 (KU310664), B3x2.4 (KU310665), B3x2.5 (KU310666). These sequences are hereby incorporated by reference.

[0095] A cell is provided comprising therein a recombinant HSV-2 genome as described herein.

[0096] Also provided is a vaccine composition comprising the recombinant HSV-2 virus as described herein. In an embodiment, the vaccine comprises an immunological adjuvant. In an embodiment, the vaccine does not comprise an immunological adjuvant. In an embodiment of the vaccine, compositions or pharmaceutical compositions described herein comprising a recombinant HSV-2, the HSV-2 is live-attenuated.

[0097] Also provided is a composition comprising the recombinant HSV-2 virus as described herein, wherein the genome of the virus or virion comprises at least a deletion of

a second gene, wherein the second gene is necessary for HSV-2 viral replication or virulence.

[0098] A pharmaceutical composition comprising the recombinant HSV-2 virus as described herein, and a pharmaceutically acceptable carrier.

[0099] In an embodiment, the composition or pharmaceutical composition or vaccine is formulated so that it is suitable for subcutaneous administration to a human subject. In an embodiment, the composition or pharmaceutical composition or vaccine is formulated so that it is suitable for oral administration to a human subject. In an embodiment, the composition or pharmaceutical composition or vaccine is formulated so that it is suitable for intravaginal administration to a human subject. In an embodiment, the composition or pharmaceutical composition or vaccine is formulated so that it is suitable for intra-muscular, intra-nasal, or mucosal administration to a human subject. In embodiments of the methods herein, and embodiments of the composition or pharmaceutical composition or vaccine formulations herein, administration can be auricular, buccal, conjunctival, cutaneous, subcutaneous, endocervical, endosinusial, endotracheal, enteral, epidural, via hemodialysis, interstitial, intrabdominal, intraamniotic, intra-arterial, intra-articular, intrabiliary, intrabronchial, intrabursal, intracardiac, intracartilaginous, intracaudal, intracavernous, intracavitary, intracerebral, intracisternal, intracorneal, intracoronary, intradermal, intradiscal, intraductal, intraepidermal, intraesophagus, intragastric, intravaginal, intragingival, intraileal, intraluminal, intralesional, intralymphatic, intramedullary, intrameningeal, intramuscular, intraocular, intraovarian, intraepicardial, intraperitoneal, intrapleural, intraprostatic, intrapulmonary, intrasinal, intraspinal, intrasynovial, intratendinous, intratesticular, intrathecal, intrathoracic, intratubular, intratumor, intratympanic, intrauterine, intravascular, intravenous, intraventricular, intravesical, intravitreal, laryngeal, nasal, nasogastric, ophthalmic, oral, oropharyngeal, parenteral, percutaneous, periarticular, peridural, rectal, inhalationally, retrobulbar, subarachnoid, subconjuctival, sublingual, submucosal, topically, transdermal, transmucosal, transplacental, transtracheal, ureteral, urethral, or vaginal. A combination comprising at least one of the foregoing routes of administration can also be used.

[00100] Also provided is a method of eliciting an immune response in a subject, the method comprising administering to the subject an amount of (i) the recombinant HSV-2 virus as described herein in an amount effective to elicit an immune response in a subject.

[00101] In an embodiment, the HSV-2 glycoprotein D-encoding gene is an HSV-2 US6 gene. In an embodiment, the HSV-2 recombinant virus encodes a heterologous surface glycoprotein. In an embodiment the heterologous surface glycoprotein is an HSV-1 gD. In an embodiment, the HSV-2 recombinant virus comprises a non-genotypically encoded HSV-1 gD also encodes a heterologous surface glycoprotein that is not a herpesvirus glycoprotein and/or is and not involved in herpesviridae infection, and which is encoded by a transgene that has been inserted into the genome of the recombinant HSV-2. In an embodiment, the genome of the recombinant HSV-2 does not encode any herpes virus gD. In an embodiment, the surface glycoprotein is present on a lipid bilayer of the virus by way of infecting a cell with a recombinant HSV-2 having a deletion of an HSV-2 glycoprotein D-encoding gene, wherein the cell is or has been transfected to express the surface glycoprotein on a cell membrane thereof, and wherein the recombinant HSV-2 comprising the surface glycoprotein present on a lipid bilayer is produced from the cell. In an embodiment the host cell is a HSV-1 gD complementing cell. In an embodiment, the host cell encodes an HSV-1 gD under the endogenous gene promoter. In an embodiment the host cell is a HSV-1 gD complementing VD60 cell. (See, e.g., Ligas et al., J Virol. 1988 May;62(5):1486-94, hereby incorporated by reference).

[00102] Also provided is a cell comprising therein a recombinant virus as described herein, wherein the cell is not present in a human being.

[00103] A vaccine composition comprising a recombinant virus as described herein. In an embodiment of the vaccine composition, the vaccine composition comprises an immunological adjuvant.

[00104] Also provided is a composition comprising a recombinant virus as described herein, wherein the genome of the virus comprises at least a deletion of a second gene, wherein the second gene is necessary for HSV-2 viral replication.

[00105] In an embodiment, the recombinant virus as described herein does not comprise a deletion of a second gene.

[00106] Reducing the likelihood of a viral infection is understood to mean amelioration of the extent of development of the relevant disease or chances of infection in a subject treated with the virus, vaccine or compositions described herein, as compared to an untreated subject.

[00107] In an embodiment of the methods herein for immunizing, vaccinating or eliciting an immune response, passive transfer of the virus or the antibodies or immune factors

induced thereby may be effected from one subject to another. The relevant product may be treated after obtention from one subject before administration to a second subject. In a preferred embodiment of the inventions described herein, the subject is a mammalian subject. In an embodiment, the mammalian subject is a human subject.

[00108] In an embodiment, vaccinating a subject with an antigen elicits a humoral immune response to that antigen in the subject. A vaccinated individual is usually able to mount a more efficacious immune response to a subsequent challenge from a pathogen comprising that antigen than they would be able to prior to vaccination.

[00109] In an embodiment of the methods described herein, the subject has not yet been infected with influenza virus. In an embodiment of the methods described herein, the subject has not yet been infected with HIV. In an embodiment of the methods described herein, the subject has been infected with influenza virus. In an embodiment of the methods described herein, the subject has been infected with HIV.

[00110] In embodiments, the influenza infection is a human influenza A infection. In embodiments, the influenza infection is a human influenza B infection. In embodiments, the HIV infection is an HIV-1 infection. In embodiments, the HIV infection is an HIV-2 infection.

[00111] "Codon optimization" is defined as modifying a nucleic acid sequence for enhanced expression in the cells of interest, e.g. human, by replacing at least one, more than one, or a significant number, of codons of the native sequence with codons that are more frequently or most frequently used in the genes of that vertebrate. Various species exhibit particular bias for certain codons of a particular amino acid. In one aspect, the present invention relates to codon optimized inserts, nucleic acids or vectors, or host cells comprising such.

[00112] Also provided is a method of quantitating a rate or amount of antibody-dependent cell-mediated killing (ADCK) in a population of cells comprising infecting a plurality of cells of the population of cell with a fluorescent protein-expressing recombinant HSV-2 that comprises a genome deleted for the gene encoding HSV-2 gD, under conditions permitting expression of the fluorescent protein in the cells, contacting the plurality of infected cells with an antibody-containing solution and a population of immune cells, and quantitating at one or more time points the amount of cells exhibiting fluorescent protein fluorescence and, optionally, one or more markers, so as to quantitate over time the amount

of live infected cells, so as to thereby quantitating the rate or amount of ADCK in the population of immune cells.

[00113] Also provided is a method of quantitating a rate or amount of antibody-dependent cell-mediated killing (ADCK) in a population of cells comprising infecting a plurality of cells of the population of cells with a fluorescent protein-expression expressing recombinant HSV-2 that comprises a genome deleted for the gene encoding HSV-2 gD, under conditions permitting expression of the fluorescent protein in the cells, contacting the plurality of infected cells with an antibody-containing solution, and quantitating at one or more time points the amount of cells exhibiting fluorescent protein fluorescence and, optionally, one or more markers, so as to quantitate over time the amount of live infected cells, so as to thereby quantitating the rate or amount of ADCK in the population of cells.

[00114] In embodiments, the recombinant HSV-2 is made by a process as described herein.

[00115] In embodiments, the method is performed *in vitro.* In embodiments, the population of immune cells comprises a population of macrophages. In embodiments, the macrophages are human. In embodiments, the antibody-containing solution comprises serum. In embodiments, the fluorescent protein is as described hereinabove. In an embodiment, the fluorescent protein is Red Fluorescent Protein. In an embodiment, only the cells quantitated as RFP^{high} are considered live. In an embodiment, the plurality of cells are RFP^{high} if they express RFP above the mean intensity of an RFp-expressing cell of the infected population of cells. In embodiments, the method is performed with the population of immune cells present at effector:target ratio of 5:1 or greater. In embodiments, the method is performed with the population of immune cells present at effector:target ratio of 10:1 or greater.

[00116] In embodiments, the amount of cells exhibiting fluorescent protein fluorescence and, optionally, one or more markers, is measured by fluorescence-activated cell sorting (FACS). In embodiments, the amount of cells exhibiting fluorescent protein fluorescence and, optionally, one or more markers, is measured by fluorescence spectrometer, fluorescence microplate reader, and fluorescence microscopy or fluorescence plate reader. In embodiments, the one or more markers comprise a cell membrane marker and/or a live/dead marker.

[00117] In embodiments, the method further comprises quantitating at one or more time points the amount of cells exhibiting fluorescent protein fluorescence and, optionally, one or

more markers, in a control population of infected cells otherwise identical, but not contacted with an antibody-containing solution and comparing the amount or rate quantitated to that quantitated for the population of cells contacted with the antibody-containing solution.

[00118] In other embodiments of the invention relating to assays for ADCK, instead of a fluorescent protein the marker in the recombinant HSV-2 ΔgD-2 can be a beta galactosidase or an alkaline phosphatase. Thus, the methods, processes and compositions disclosed herein can comprise, *mutatis mutandis*, recombinant HSV-2 ΔgD-2 comprising a nucleic acid in the genome thereof encoding beta galactosidase or an alkaline phosphatase.

[00119] "And/or" as used herein, for example, with option A and/or option B, encompasses the separate embodiments of (i) option A, (ii) option B, and (iii) option A plus option B.

[00120] All combinations of the various elements described herein are within the scope of the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

[00121] This invention may be better understood from the Examples, which follow.

EXAMPLES

Example 1

[00122] An engineered HSV-2 virus that replaces the gD gene (ΔgD-2) with a gene that strongly expresses the red fluorescent protein (RFP) was constructed. Once achieved, this provided an improved screen for identifying and obtaining new recombinants. The ΔgD-2*::*RFP recombinant was made from a ΔgD-2 that showed protection. The red fluorescent ΔgD-2*:*RFP, referred to herein also as B^3x 2.8 or ΔgD-2::$P_{EF1\alpha}$-RFP, retains the capacity of the original ΔgD-2 to elicit protective immunity against HSV infection but also appears to have unaltered *in vitro* replication kinetics. Moreover, ΔgD-2*::*RFP possesses significant advantages over HSV-2 ΔgD::GFP virus for making recombinants. Since the *RFP* gene is fused to a highly efficient promoter, for example promoter of the EF1α (Elongation Factor 1 α) gene, its expression is easily detected using a fluorescent microscope. In addition, visualization of red fluorescence in cells infected with ΔgD-2*::*RFP does not have the background found with the green fluorescent ΔgD-2. This property enables unambiguous identification of recombinant viruses in which the Δ*gD-2*::$P_{EF1\alpha}$-*RFP* allele is replaced by other allelic exchange substrates.

[00123] Construction of the HSV-2 ΔgD-/+gD-1 virus (hereafter called "ΔgD-2") using HSV-2(G) as the backbone was accomplished as detailed below. To make the allelic exchange constructs, an expression construct was generated by Gibson cloning into an *E. coli* plasmid, cloning the expression construct in between the SpeI and BclI sites of a shuttle (for example, cosmid pYUB2169) that contains 12 kb of the HSV-2(G) virus spanning from US1 To US9, electroporated the *PacI* restriction fragment from the pYUB2169 recombinant alongside ΔgD-2::RFP DNA into VD60 cells, and screening the resulting plaques for loss of red fluorescence. (See FIG. 1).

[00124] This system was used to generate two sets of recombinant ΔgD-2 strains containing hemagglutinin (HA) antigens derived from the influenza A virus (IAV) strain A/Puerto Rico/8/1934 (PR8). The first set of recombinants contain chimeric gD::HA genes recombined into the US6 region of ΔgD-2 through the use of, for example, pYUB2169. These recombinant genes were made up of the extracellular domain of PR8 HA fused to the transmembrane, cytosolic, and signal sequence domains of HSV-2 (G) gD. The second set of recombinants contain modified or unmodified PR8 HA genes fused to an upstream CMV promoter and downstream SV40 polyadenylation signal. These cassettes were inserted into the US6 region of ΔgD-2 using a modified pYUB2169 identified as pBRL812. Each set of recombinants contained three type of HA genes:

FL HA nOP – Full-length extracellular domain hemagglutinin, non-codon optimized.

HL HA nOP – Headless extracellular domain hemagglutinin, non-codon optimized.

HL HA OPT – Headless extracellular domain hemagglutinin, codon-optimized.

[00125] The following constructs were made:

[00126] ΔgD-2::RFP (ΔgD-2::PEF1α-RFP) was constructed by (1) introducing a kanamycin marker in RFP plasmid ptwB, (2) amplifying the *kan* marker and RFP (dTomato) with oligos having ~50bp homologous sequence upstream and downstream of US6, (3) recombining in DY331 cells, then adding the PCR product followed by transformation with cosmid pYUB2156. (4) The *kan* marker was removed by restriction digest and ligation. (5) HSV2(G) genomic DNA and pYUB2167 were co-transfected in VD60 cells to generate ΔgD-2::RFP by allelic exchange.

[00127] ΔgD-2 was obtained by co-transfection of cosmid pYUB2163 with the ΔgD-2::RFP genome in VD60 cells to generate the ΔgD-2 genome by allelic exchange. This genome is free of all markers and antibiotic resistance genes. The resultant unmarked virus

was sorted based on lack of RFP expression. RFP-negative plaques were purified three times and the lack of the RFP gene was verified by PCR and sequencing.

[00128] ΔgD-2::FL HA nOP was constructed by (1) Gibson cloning the cytosolic, transmembrane, and signal sequence domains of HSV-2 (G) gD to the extracellular domain of custom synthesized PR8 *HA* (Genscript, Piscataway, NJ) in the pYUB2169 plasmid and (2) co-transfecting the resultant plasmid, pBJJ1, with the ΔgD-2:RFP genome in VD60 cells to generate the desired recombinant by allelic exchange.

[00129] ΔgD-2::HL HA nOP was constructed similarly to ΔgD-2::FL HA nOP, but using a custom synthesized PR8 *HA* gene where the HA1 head domain was replaced with 4 glycine residues (Genscript). The plasmid used for transfection was called pBJJ2.

[00130] ΔgD-2::HL HA OPT was constructed similarly to ΔgD-2::HL HA nOP, but using a custom synthesized PR8 HA gene where the HA1 head domain was replaced with 4 glycine residues and every codon which had less than 9.5% representation in HSV-2 (G) *gD* for its cognate amino acid was replaced with the codon most abundant in HSV-2 (G) *gD* (Genscript). The plasmid used for transfection was called pBJJ4.

[00131] ΔgD-2::P_{CMV}-FL HA nOP was constructed by (1) restriction cloning the full length PR8 *HA* gene (Genscript) in between the xbaI and HindIII restriction sites of pEGFP-N1 (Addgene, Cambridge, MA) (2) restriction cloning the P_{CMV}-FL HA nOP::SV40 polyA cassette from the resultant plasmid into the SpeI and BclI sites of pBRL812. The resulting plasmid, pJHA1, was cut with PacI and AsiSI and co-transfected with the ΔgD-2::RFP genome in VD60 cells to generate the desired recombinant by allelic exchange.

[00132] ΔgD-2::P_{CMV}-HL HA nOP was constructed similarly to ΔgD-2::P_{CMV}-FL HA *nOP* but using a custom synthesized PR8 *HA* gene where the HA1 head domain was replaced with 4 glycine residues (Genscript). The plasmid used for transfection was called pJHA2.

[00133] ΔgD-2::P_{CMV}-HL HA OPT was constructed similarly to ΔgD-2::P_{CMV}-FL HA nOP but using a custom synthesized PR8 *HA* gene where the HA1 head domain was replaced with 4 glycine residues and every codon which had less than 9.5% representation in HSV-2 (G) gD for its cognate amino acid was replaced with the codon most abundant in HSV-2 (G) gD (Genscript). The plasmid used for transfection was called pJHA4.

Table 2: HSV-2 Viruses Generated

Stock #	Parent	Genotype	How Constructed	Reference
B^3x 2.6	HSV-2 G	Wild-type	N/A	ATCC
B^3x 2.7	HSV-2 G	ΔgD-2::GFP	Allelic Exchange w G	Petro, Gonzalez et al. *Elife* Petro et al. *JCI*
B^3x 2.8	B^3x 2.7	ΔgD-2::RFP (also referred to as ΔgD-2::$P_{EF1\alpha}$-RFP)	Allelic Exchange w B^3x 2.6	Disclosed herein
B^3x 2.9	HSV-2 G	ΔgD-2	Allelic Exchange w B^3x 2.8 (Unmarked ΔgD-2)	Disclosed herein
B^3x 2.10	HSV-2 G	ΔgD-2::HA_{PR8}	Allelic Exchange w B^3x 2.8	Disclosed herein
B^3x 2.11	HSV-2 G	ΔgD-2:: $HA_{PR8\text{-}STALK}$	Allelic Exchange w B^3x 2.8	Disclosed herein
$\beta^3\chi$1	HSV-2 G	ΔgD-2::FL HA nOP	Allelic Exchange w B^3x 2.8	Disclosed herein
$\beta^3\chi$2	HSV-2 G	ΔgD-2::HL HA nOP	Allelic Exchange w B^3x 2.8	Disclosed herein
$\beta^3\chi$3	HSV-2 G	ΔgD-2::HL HA OPT	Allelic Exchange w B^3x 2.8	Disclosed herein
$\beta^3\chi$4	HSV-2 G	ΔgD-2::P_{CMV}-FL HA nOP	Allelic Exchange w B^3x 2.8	Disclosed herein
$\beta^3\chi$5	HSV-2 G	ΔgD-2::P_{CMV}-HL HA nOP	Allelic Exchange w B^3x 2.8	Disclosed herein
$\beta^3\chi$6	HSV-2 G	ΔgD-2::P_{CMV}-HL HA OPT	Allelic Exchange w B^3x 2.8	Disclosed herein

[00134] Whole genome sequencing was used to verify the deletion of US6. To test immunogenicity, mice were vaccinated on Day 0 (d0) and d21, then sera was taken on d40.

ΔgD-2 elicited anti-HSV antibodies that are similar to those obtained with ΔgD-2::GFP (See FIG. 2).

[00135] Mechanistically, it seems that the most important correlates of protection are ADCC and ADCP. Sera from ΔgD-2 vaccinated mice induced significantly more ADCC/ADCP immunity than sera from ΔgD-2::GFP vaccinated mice (FIG. 3). The CD8 and CD4 T-cell responses were similar at 40 days using the same vaccination strategy (FIG. 4).

[00136] ΔgD-2 vaccination protects mice against challenge with a wild-type HSV-2 4674, which is a clinical isolate obtained from the virology laboratory at Montefiore Hospital, Bronx, NY, as well as the GFP-marked construct. Mice were vaccinated with either the wild-type HSV-2 or the GFP-marked construct virus on d0 and d21. Twenty-one days after boost, mice were challenged subcutaneously with 10X LD90 HSV-2 4674 and followed for skin lesion changes, presence of virus in the dorsal root ganglia, body weight, and survival. The unmarked virus behaved nearly identically by all measures (FIG. 5).

Materials and Methods

[00137] HSV-1(17) (Brown et al., 1973), HSV-2(G) (Ejercito et al., 1968), HSV-1(F) (Ejercito et al., 1968), and HSV-2(333)ZAG (Nixon et al., 2013), a recombinant virus expressing the green fluorescent protein (GFP) were propagated on Vero cells. HSV-2 4674 (Nixon et al., 2013) was propagated on HaCAT cells. VD60 cells (Vero cells encoding gD-1 under the endogenous gene promoter (Ligas and Johnson, 1988)) were passaged in DMEM supplemented with 10% fetal bovine serum. Stocks of HSV-2 ΔgD-2 (ΔgD-/+gD-1) virus were propagated on complementing VD60 cells and titered on VD60 and Vero cells. Concentrated viral stocks were stored at -80°C and diluted in PBS to the desired concentration when needed.

[00138] Construction of ΔgD-2::GFP. Plasmid pcDNA3-eGFP (13031; Addgene, Cambridge, MA, USA) was used as a template to PCR amplify the pCMV-eGFP-Neo^r and OriE-Amp^r regions flanked by Van91I restriction enzyme sites. The pCMV-eGFP-Neo^r region was PCR-amplified using primers Fwd-pCMV and Rev-NeoR-Term (see Table 3 for a list of primers). The OriE-Ampr region was PCR amplified using primers Fwd-Origin and Rev-AmpR.

[00139] In parallel, genomic regions flanking the left and right of the US6 gene (gD) in HSV-2 were PCR amplified using purified viral DNA (HSV-2 strain 4674) as a template

and primers LL-V91I-US6 plus LR-V91I-US6 for the left homology arm and primers RL-V91I-US6 and RR-V91I-US6 for the right homology arm (see Table 3 for sequence alignment). All four PCR fragments were gel purified, digested with Van91I (Fermentas Molecular Biology Tools, Thermo Scientific, West Palm Beach, FL, USA), ligated with Quick-Ligase [New England Biolabs (NEB), Ipswich, MA, USA], and transformed into NEB 5-α competent cells.

[00140] The resulting plasmid (eKO2-US6) was sequence verified and extracted from *E. coli* using an endotoxin-free miniprep kit (MO-BIO Laboratories, Carlsbad, CA, USA). HSV-2 DNA (1 μg) was co-transfected with 100 ng of eKO2-US6 into VD60 cells using Effectene (Qiagen, Valencia, CA, USA), according to the manufacturer recommendations.

[00141] At 4 d after transfection, plates were screened for green plaques and supernatants were collected and overlaid on fresh VD60 cells for 1 h, then washed and covered with 4% low-melting agarose prepared in Optimem (Invitrogen, Carlsbad, CA, USA). Single green fluorescent plaques were picked and purified 3 times using this method. Viral stocks were grown on VD60 cells, and noncomplemented virus was generated by harvesting infected cell lysates from Vero cells, as described for HSV-1 gD-deletion viruses (Ligas, 1988).

[00142] Genotypic confirmation of the gD deletion in ΔgD-2::GFP was performed by PCR. A primer set was used to confirm the presence of wild-type (WT) and ΔgD-2 virus DNA in the samples (primers RL-V91I-US6 and RL-V91IUS6), while another set of primers (Neo-Out and US8-Out) was used to amplify a DNA region comprising eK02-US6 and the genomic target region. To confirm deletion of gD expression, Vero or VD60 cells were infected with parental WT or ΔgD-2 virus (grown on VD60 cells and thus competent for entry) at a multiplicity of infection (MOI) of 10 plaque-forming units (PFU)/cell (based on VD60 titer). After 1-h incubation, cells were washed twice with PBS, incubated in Optimem for 48 h at 37°C, and harvested and evaluated for gD expression by Western blot.

Table 3. Primers used in constructing a gD-2 deletion virus.

Region amplified	Primer Name	Sequence (5' to 3')
a. Primers for constructing eKO2-US6 (*Van91I sites are underlined)*		
pCMV-eGFP-Neomycin resistance	*Fwd-pCMV*	TTT TTT TTC CAA GAA ATG GAG GCC TAC CCG GGT TGA CAT TGA TTA TTG ACT AGT TAT TAA TAG TAA TC (SEQ ID NO:3)
	Rev-NeoR-	TTT TTT TTC CAA TCT ATG GAG CCC CAG CTG

	Term	GTT CTT TCC (SEQ ID NO:4)
pUC Origin-Ampicillin resistance	*Fwd-Origin*	TTT TTT TTC CAA TTT ATG GGC TGG GCT GTG TGC ACG AAC C (SEQ ID NO:5)
	Rev-AmpR	TTT TTT TTC CAA AAG ATG GGC AGC GCA AAA CGC CTA ACC CTA AG (SEQ ID NO:6)
Left US6 homology arm	*LL-V91I-US6*	TTT TTT TTC CAT AAA TTG GAA AGG GAA CAG CGA CCA AAT GTC AC (SEQ ID NO:7)
Right US6 homology arm	*LR-V91I-US6*	TTT TTT TTC CAT TTC TTG GTG ATA CGC GAT GCA CAC GAA AAA CG (SEQ ID NO:8)
	RL-V91I-US6	TTT TTT TTC CAT AGA TTG GTT CCC CGC TCC CGT GTA CC (SEQ ID NO:9)
	RR-V91I-US6	TTT TTT TTC CAT CTT TTG GCG GGG GCG CCT GTA TCG G (SEQ ID NO:10)
b. Primers for verifying gene deletion		
WT and ΔgD virus	*RL-V91I-US6*	TTT TTT TTC CAT AGA TTG GTT CCC CGC TCC CGT GTA CC (SEQ ID NO:9)
	RR-V91I-US6	TTT TTT TTC CAT CTT TTG GCG GGG GCG CCT GTA TCG G (SEQ ID NO:10)
ΔgD virus	*Neo-Out*	GTA TAC CGT CGA CCT CTA GC (SEQ ID NO:11)
	US8-Out	GGT GAC TTG GTG CGC CGC C (SEQ ID NO:12)

[00143] Sequence homologies for the PCR fragments used to construct the eKO2-US6 plasmid. Searching the published Addgene pcDNA3-eGFP (id:13031, 6159bp) sequence for the pCMV-eGFP-Neomycin resistance primers using Blast2 yields a 1819bp fragment:

```
Query  10   CGGGTTGACATTGATTATTGACTAGTTATTAATAGTAATC  49 (SEQ ID NO:13)
            || ||||||||||||||||||||||||||||||||||||
Sbjct  229  CGCGTTGACATTGATTATTGACTAGTTATTAATAGTAATC  268    Plus strand

Query  1     AGCCCCAGCTGGTTCTTTCC  20 (SEQ ID NO:14)
              ||||||||||||||||||||
Sbjct  2010  AGCCCCAGCTGGTTCTTTCC  1991                    Minus strand
```

[00144] Searching the pcDNA3-eGFP sequence for the pUC Origin-Ampicillin resistance primers yields a 1754bp fragment:

```
Query  1     GCTGGGCTGTGTGCACGAACC  21  (SEQ ID NO:15)
             |||||||||||||||||||||
Sbjct  4647  GCTGGGCTGTGTGCACGAACC  4667            Plus strand

Query  1    GCAGCGCAAAACGCCTAACCCTAAG  25 (SEQ ID NO:16)
            |||||||||||||||||||||||||
Sbjct  204  GCAGCGCAAAACGCCTAACCCTAAG  180           Minus strand.
```

[00145] Searching the blast.ncbi.nlm.nih.gov/Blast.cgi database for Left US6 homology arm of Herpes simplex virus (type 2) (taxid:10310) returned sequence from the Human HSV-2 strain HG52, complete genome:

BLAST for Left-US6 homology arm yields a 991bp homologous region, 1029bp fragment:

```
Query  1       AAAGGGAACAGCGACCAAATGTCAC  25 (SEQ ID NO:17)
               |||||||||||||||||||||||||
Sbjct  140020  AAAGGGAACAGCGACCAAATGTCAC  140044     Plus strand

Query  1       TGATACGCGATGCACACGAAAAACG  25  (SEQ ID NO:18)
               |||||||||||||||||||||||||
Sbjct  141011  TGATACGCGATGCACACGAAAAACG  140987     Minus strand.
```

[00146] BLAST for Right-US6 homology arm yields a 1072bp homologous region, 1110bp fragment:

```
Query  1       TTCCCCGCTCCCGTGTACC  19        (SEQ ID NO:19)
               |||||||||||||||||||
Sbjct  142204  TTCCCCGCTCCCGTGTACC  142222          Plus strand

Query  1       CGGGGGCGCCTGTATCGG  18         (SEQ ID NO:20)
               ||||||||||||||||||
Sbjct  143276  CGGGGGCGCCTGTATCGG  143259       Minus strand
```

[00147] Table 4: Codons replaced in custom synthesized codon-optimized HA genes. When synthesizing *HA* genes, versions were designed that replaced codons in PR8 *HA* that had less than 9.5% representation in HSV-2 (G) *gD* with codons for their respective amino acid that had the highest representation in HSV-2 (G) *gD*.

Original Codon	Prevalence in HA	Prevalence in gD	Altered Codon	Prevalence in gD	Replacements Made
ATA	38.89%	4.35%	ATC	86.96%	14
ATT	30.56%	8.70%	ATC	86.96%	11
GTA	28.53%	4.00%	GTC	40.00%	8
CTT	9.62%	5.13%	CTG	43.59%	5
TTA	11.54%	7.69%	CTG	43.59%	6
TCT	28.13%	7.69%	TCG	69.23%	9
TCA	53.13%	0.00%	TCG	69.23%	17
CCT	5.00%	0.00%	CCC	65.12%	1
CCA	50.00%	6.98%	CCC	65.12%	10
ACT	23.33%	4.35%	ACG	39.13%	7
ACA	46.67%	4.35%	ACC	52.17%	14
GCA	45.45%	7.32%	GCC	60.98%	15
CAT	53.85%	9.09%	CAC	90.91%	7
TGT	62.50%	0.00%	TGC	100.0%	10
AGT	53.33%	8.33%	AGC	91.67%	8
AGA	52.17%	0.00%	CGC	58.33%	12
AGG	47.83%	0.00%	CGC	58.33%	11

[00148] The presence of chimeric gD::HA in recombinant viruses was verified by PCR (see Fig. 7). The lanes from left to right are as follows: Lane 1; ΔgD-2genomic DNA, Lane 2; ΔgD-2::FL HA nOP genomic DNA, Lane 3; pBJJ1 plasmid DNA, Lane 4; ΔgD-2genomic DNA, Lane 5; ΔgD-2::HL HA nOP genomic DNA, and Lane 6; pBJJ2 plasmid

DNA. Primers used for PCR amplification were located just upstream and downstream of the HA extracellular domains. No exogenous promoter was inserted into the expression cassette. As a result, chimeric gene expression was regulated by the endogenous promoter. Sanger sequencing was used to verify the proper construction of chimeric gD::HA genes in recombinant viruses. Upon genetic verification, 6-8 week old C57BL/6 mice were prime-boost vaccinated at days 0 and 21 with ΔgD-2::FL HA nOP or ΔgD-2::HL HA OPT in parallel with the parental ΔgD-2::RFP strain and a control group mock-vaccinated with VD60 cell lysates. 21 days post-boost, the mice were skin challenged with a 10x LD90 of HSV-2 4674 to determine whether anti-HSV immunity had been compromised.

[00149] Mice that were vaccinated with ΔgD-2::FL HA nOP and ΔgD-2::HL HA OPT are fully protected against HSV-2 challenge but do not form anti-HA IgGs (see FIG. 8). Mice were prime-boost vaccinated on days 0 and 21 with a control VD60 cell lysate or $1x10^6$ PFU of ΔgD-2::FL HA nOP, ΔgD-2::HL HA OPT, or ΔgD-2::RFP. At day 42, mice were challenged with a 10x LD90 of HSV-2 4674. Mice vaccinated with ΔgD-2::FL HA nOP and ΔgD-2::HL HA OPT were fully protected from challenge. (See FIG. 8A) At day 40 post-prime, mice were bled to look at serum antibodies. ELISAs performed against soluble PR8 HA show the absence of HA-specific IgGs in mice. (See FIG. 8B)

[00150] Construction of ΔgD-2::P_{CMV}-HA viruses and HA expression. It was investigated if it would be more immunogenic to insert non-chimeric HA genes into the ΔgD genome in cassettes containing a constitutive promoter and poly-adenylation sequence. To accomplish this, the synthesized PR8 HA genes were restriction cloned into expression plasmid pEGFP-N1 (Addgene). HA genes were inserted in between xbaI and HindIII sites and replaced EGFP in the plasmid.

[00151] Mice were vaccinated with ΔgD-2::P_{CMV}-FL HA nOP. The mice were prime vaccinated with $5x10^6$ PFU of each virus (See FIG. 18 legend for the different viruses used). n=5 mice/group, except 10 mice for ΔgD-2::FL HA nOP. Five (5) of the ΔgD-2::FL HA nOPmice were boosted with a stalk HA expressing ΔgD-2, 5 μg/mL of purified PR8 HA was used as the source of antigen. A goat anti-mouse Ig Ab was used for the total Ab ELISA. Day 14 post-prime anti-PR8 HA ELISA showed serum from mice vaccinated with stalk HA (ΔgD-2::FL HA nOP) showed an overall Ab response, but it did not register above background on the isotype ELISAs. (See FIG. 18) Importantly, vaccination with ΔgD-2::P_{CMV}-FL HA nOP (β3χ4) yielded significantly more IgG2c than IgG1 (See FIG. 19). Vaccination with the full-length HA induced the largest anti-HA response and this response

is predominantly IgG2c. This is consistent with identical IgG isotype responses being obtainable with the recombinant virus vaccine to exogenous antigens as to endogenous HSV proteins.

[00152] ΔgD-2::P_{CMV}-*HA* recombinant viruses all express *HA*. As shown in FIG. 12, VD60 cells were infected with 3 MOI of ΔgD-2::P_{CMV}-FL HA nOP, ΔgD-2::P_{CMV}-HL HA nOP, or ΔgD-2::P_{CMV}-HL HA OPT. At 16 hours post-infection, cells were harvested and stained for HSV-protein and HA expression. HSV protein expression was measured using serum from mice vaccinated with ΔgD-2::RFP. HA expression was measured using monoclonal anti-HA stalk IgG C179. Cells were stained with either cell permeable or cell impermeable methods. Statistics were calculated by student's *t* test of geometric means between control fluorescence and fluorescence in wells stained for HA expression. Only $\beta^3\chi4$ induced high levels of HA expression in infected cell membranes, but all three viruses induced expression as measured by cell-permeable staining.

[00153] To determine if the insertion of full-length PR8 HA into ΔgD-2 would introduce a novel route of cell entry, Vero cells were infected with 0.01 MOI of ΔgD-2::P_{CMV}-FL HA nOP or ΔgD-2::$P_{EF1\alpha}$-RFP. At 120 hours post-infection, there were no signs of productive infection. Additionally, 5 C57BL/6 mice were given subcutaneous injections with $5x10^6$ PFU of ΔgD-2::P_{CMV}-FL HA nOP and monitored daily for one week. They showed no signs of disease or distress. Additionally, an in vitro assay was conducted in which non-complementing Vero cells were infected with titrations of either ΔgD-2::P_{CMV}-FL HA nOP and ΔgD-2::PEF1α-RFP. At 70 hours post-infection, the cells were fixed and stained for HSV-2 glycoprotein B (gB). Staining shows the presence of isolated infected cells at a 10^{-3} dilution of each virus, but no evidence of cell-to-cell viral spread (See FIG. 13). This is not surprising, given that the influenza A and B proton pump genes, both named M2, are required the mediate release of virions from the endosome. Since β3χ4-6 do not contain either proton pump gene, they lack a mechanism to exit the endosome of cells after entry.

Example 2

[00154] Designing the HIV constructs to express in HSV-2 *ΔgD* virus: Producing a vaccine vector in which a glycoprotein antigen from HIV would be expressed from the ADCC-inducing HSV-2 *ΔgD* vector was investigated. The rationale for using this vector is that it elicits non-neutralizing, ADCC-inducing IgG antibodies against HSV, and this type of immune response has been correlated with protection in the only HIV vaccine trial to

show any efficacy thus far (Haynes, Gilbert et al. 2012). For the glycoprotein antigen, the transmitted/founder clone of Env gp145 was chosen, which lacks the cytoplasmic tail, from donor CH505 in the CHAVI001 acute HIV-1 infection cohort, as this well-characterized HIV-1 clade C glycoprotein is thought to be representative of those that pass the bottleneck of infection in a region of high HIV prevalence (Liao, Lynch et al. 2013). HIV Env is not expressed particularly well in the context of natural infection and often not expressed well exogenously, so steps were investigated to enhance antigen expression in our construct. Env gp145 was found to be incorporated more efficiently into virus like particles (VLPs) than full length, and replacing the Env signal peptide and transmembrane domains with corresponding domains from host proteins or other viral glycoproteins increased incorporation into VLPs further (Wang, Liu et al. 2007). To provide efficient incorporation of HIV Env into HSV VLPs, a chimera of the ectodomain of HIV Env with the signal peptide and transmembrane cytoplasmic tail of HSV-2 gD was constructed. The signal peptide of HSV2 gD is 25 residues in length and the ectodomain is 306 residues total (Eisenberg, Long et al. 1984, Nicola, Willis et al. 1996). The allelic exchange construct was generated by Gibson assembly (Gibson, Young et al. 2009). Briefly, oligonucleotide primers were synthesized to amplify from HSV2 strain G genomic DNA arms of homologous sequence ~800bp 5' to the 25th codon of HSV2 US6 and ~800 bp 3' to the 306th codon of HSV2 US6. The insert was amplified from the CH505 TF gp145 expression plasmid HV1300631 (gift of Huaxin Liao, Duke University) with primers that encompassed the 30th codon to codon 680. The fragments were cloned into pUC19 between EcoRI and BamHI restriction sites.

Example 3

Introduction

[00155] Functional *in vitro* macrophage antibody-dependent cell-mediated killing (ADCK) assay using an RFP-expressing HSV-2 Δ*gD* strain (as made in Example 1): The single-cycle herpes simplex virus type 2 (HSV-2) strain deleted in glycoprotein D (ΔgD-2) elicits sterilizing anti-HSV immunity by inducing antibodies that bind and activate Fcγ-receptors (FcγRs). Murine FcγRIV is highly activated in the presence of serum from vaccinated mice and is expressed on macrophages, monocytes, and neutrophils. The precise mechanisms of cell killing through FcγRs are not well understood and additional tools are needed. FcγR-binding antibodies mediate killing of HSV-infected cells by binding antigens

on infected cell and then binding and activating FcγRs on innate leukocytes. This precipitates antibody-dependent cell-mediated cytotoxicity and phagocytosis (ADCC and ADCP), here referred to as antibody-dependent cell-mediated killing (ADCK). Current assays face many limitations including, but not limited to, inflexible target and effector cell lines, artificial antigen presentation systems, indirect or separate outputs for ADCC and ADCP, and the use of cumbersome radioactive isotopes.

[00156] To overcome these restrictions, a quantitative *in vitro* assay was constructed to study ADCK in response to anti-HSV antibodies. A ΔgD-2 variant that highly expresses the gene for red fluorescent protein (*rfp)* was used to mark infection. RFP and cell viability markers were then used to identify live-infected target cells, the decreasing proportion of which was determined to be the result of cell killing. FACS analysis was used to quantify the decreasing proportion of live-infected target cells after co-culture with macrophages.

[00157] Using the new assay, it was shown that serum from mice vaccinated with ΔgD-2 induces significant amounts of ADCK by both immortalized macrophage cell lines and bone marrow derived macrophages (BMDMs) from both mice and guinea pigs. Live-imaging of the assay using Raw macrophages shows that killing readouts cannot easily be attributed to cytotoxicity or phagocytosis, but that the processes can occur simultaneously, underlining the importance of using an assay which measures both. Additionally, ADCK was eliminated when BMDMs from $Fc\gamma R^{-/-}$ mice were used, indicating that the primary readout is FcγR dependent and that the assay is amenable to studying ADCK in different knockout mouse strains. This assay allows for the precise study of ADCK and its associated genes and FcγRs in different species and animal models.

[00158] Traditionally, ADCC was measured by cumbersome ^{51}Cr release assays, but a number of FACS methods have been developed to measure ADCC in response to different antigens and antibodies. The collective term for these methods is Rapid fluorometric ADCC (RFADCC). In these assays, target cells are stained with a persistent membrane die and a live-dead marker that dissipates upon the initiation of apoptosis. ADCC activity is then measured as the decrease in proportion of membrane dye^+ cells that are also $live\text{-}dead^+$. In similar assays, ADCP is measured by the proportion of macrophages that are marked by the phagocytosis of fluorescent cells. However, as the data herein show, ADCC and ADCP cannot be cleanly separated by these methods, so they are referred to collectively in this text as antibody-dependent cell-mediated killing (ADCK). The prior art assays have some additional drawbacks, as they are usually restricted in their choice of antibody or effector

cell. A similar assay was used in Petro et al. 2015, but the ability of HSV to infect, replicate in, and kill both target and effector cells limited the utility of the assay for studying immune cells and their FcγRs (Petro et al., 2015).

[00159] ΔgD-2 is a single-cycle virus in non-complementing cells and was recently shown not to induce dendritic cell death *in vitro*. It was investigated whether a brightly fluorescent ΔgD-2 strain would allow for precise investigation of the cellular mechanisms of ADCK. An RFP expressing ΔgD-2 (ΔgD-2::RFP) strain was constructed and developed with an RFADCK assay that precisely measures the effector activity of macrophages on HSV-2 ΔgD-2:RFP infected cells *in vitro*. The assay was validated for both immortalized and primary cell lines using J774 cells, Raw 264.7 macrophages, and bone marrow-derived macrophages (BMDMs). Unlike current RFADCC assays, this method simulates an infectious environment, measures both ADCC and ADCP, and is profoundly flexible, allowing for the use of polyclonal animal sera and different cell lines and mouse strains.

[00160] Cells Lines: Vero (CCL-81; ATCC, Manassas, VA) and VD60 cells (Vero cells containing multiple copies of *gD*-1 under the endogenous gene promoter) were passaged in DMEM (Thermo Fisher Scientific, Waltham, MA) supplemented with 5% fetal bovine serum (FBS, Gemini Bio-Products, West Sacramento, CA) and 10 U/ml penicillin, and 10 μg/ml streptomycin sulfate (Thermo Fisher Scientific). Bone-marrow derived macrophages (BMDMs) were obtained as previously described from C57Bl/6 mice. Bone marrow precursors were stored in DMEM supplemented with 50% FBS and 10% DMSO (Sigma-Aldrich, St. Louis, MO) if not used immediately. Mouse BMDMs were differentiated using the supernatant from L929 cell cultures (ATCC). Guinea pig BMDMs were differentiated using recombinant human M-CSF (BioLegend, San Diego, CA). Raw 264.7, J774.1, and HEK 293 cells (ATCC) were passaged in DMEM supplemented with 10% FBS and 1% Pen-strep.

[00161] Creation of ΔgD-2::RFP and Virus Propagation. VD60 cells were co-transfected with HSV-2 ΔgD:GFP (ΔgD-2) genomic DNA and cosmid DNA containing 40 kB of the HSV-2 genome in which the US6 gene was replaced with *tdtomato* downstream of an EF1α promoter. Resultant virus was plaque purified three times using RFP expression as a marker of homologous recombination. The purified ΔgD-2::RFP virus was verified by PCR and sequencing.

[00162] ΔgD-2 and ΔgD-2::RFP were propagated on VD60 cell, which complement the *gD* deletion and allow for multiple rounds of replication. All viral strains were titered by serial dilution and propagation on their respective cell types.

[00163] Animals: Female C57BL/6 mice, aged 4-6 weeks, obtained from Jackson Laboratory (JAX, Bar Harbor, ME) were used to obtain serum. Male C57BL/6 mice, aged 4-6 weeks, were obtained from JAX and bone marrow cell suspensions were isolated by flushing their femurs with DMEM supplemented with 10% FBS and 1% Pen-strep. Female Hartley guinea pigs, aged 5-6 weeks, were purchased from Charles River laboratories (Wilmington, MA). Bone marrow cell suspensions were isolated by flushing femurs and tibias with DMEM supplemented with 10% FBS and 1% Pen-strep. Vaccinations were administered by subcutaneous injection. All procedures were approved by the Albert Einstein College of Medicine Institutional Animal Care and Use Committee

[00164] Immunization: Mice or guinea pigs, aged 6-8 weeks, were prime vaccinated subcutaneously with $5x10^6$ plaque forming units (PFU) of HSV-2 Δg*D::*RFP or an equal volume of VD60 cell lysate in phosphate-buffered saline (PBS) to a total volume of 200 μL. Animals were boosted with the same dose 21 days later.

[00165] Antibody-dependent cell killing assay: Bone Marrow Derived Macrophages (BMDMs), J774.1 macrophages, and Raw 264.7 macrophages were incubated in LPS (Sigma-Aldrich) for 12 hours prior to co-culture with HEK 293 cells (ATCC). HEK 293 cells were double stained with PKH67 membrane (Sigma-Aldrich) and Tag-it Violet™ (Biolegend) dyes according to manufacturer's instructions. HEK cells were infected with HSV-2 Δg*D::*RFP at a MOI of 3 in serum free DMEM 4 hours before co-culture. The infection media was removed after 3.5 hours and was replaced with a 1:5 dilution of heat-inactivated mouse or guinea pig serum collected at day 40 from mice prime-boost injected as described previously with either HSV-2 Δg*D::*RFP or VD60 cell lysate. HEK 293T cells were incubated in serum for 30 minutes at 37°C and then added to macrophage cultures in 96-well tissue culture plates (Corning Inc, New York City, NY). The co-cultures were incubated for 12 hours, then fixed and analyzed by flow cytometry on a LSRII (BD Biosciences, Franklin Lakes, NJ). For live-imaging experiments, Raw 264.7 macrophages and HEK 293 cells were co-cultured at a ratio of 10:1 on glass-bottom 96-well plates (Matrical Bioscience, Spokane WA) and imaged with an inverted NIKON Eclipse TiE microscope using NIS Elements software with deconvolution.

[00166] Statistical Analyses: Data was compiled in GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). Statistical analysis using the tests noted in the figures were also done using GraphPad Prism. Statistical significance is indicated in figures as *, $p<0.5$; ***, $p<0.001$.

Results

[00167] Inserting *rfp* into US6 of HSV-2 Δg*D* does not alter viral growth phenotype. Vero cells were infected with HSV-2 ΔgD-2::GFP at a multiplicity of infection (MOI) of 1 to determine the kinetics of GFP expression. Fluorescent microscopy was used to visualize GFP expression, which was low even at 24 hours post-infection (FIG. 14B). We replaced the GFP in the US6 locus of ΔgD-2 with an RFP under an EF1α promoter to create ΔgD-2*::*RFP (FIG. 14A). To determine the kinetics of RFP expression, non-complementing Vero cells were infected with 1 MOI of HSV-2 ΔgD-2*::*RFP or ΔgD-2. Visualization by fluorescent microscopy showed that RFP expression began in ΔgD-2::RFP at 4.5 hours post-infection and continued to increase up to 24 hours post-infection (FIG. 14C). Phase-contrast imaging showed that viral infection with ΔgD-2*::*RFP appears similar to that of the parental ΔgD-2 strain in Vero cells (FIG. 14B and 14C). Infection with ΔgD-2*::*RFP at an MOI of 3 resulted in syncytia formation in VD60 (FIG. 14E) but not Vero cells (FIG. 14D) at 12 hours post-infection, indicating that the virus maintained its single-cycle replication phenotype. The introduction of pEF1α::RFP into the *gD* locus of ΔgD-2::GFP elicited robust RFP expression while maintaining the viral kinetics and single-cycle phenotype of the parental ΔgD-2 strain.

[00168] Serum from ΔgD-2::RFP vaccinated mice induces significant ADCK in the RFADCK assay. In order to measure the levels of ADCK activity elicited by serum from ΔgD-2::RFP vaccinated mice, an *in vitro* protocol was developed based on previous RFADCC assays. Target HEK 293T cells were stained with a membrane dye and a live-dead marker and infected with ΔgD-2::RFP at an MOI of 3. Infected target cells were incubated for 3.5 hours before infection media was removed and media or mouse serum was added for an additional 30-minute incubation. The infected HEK 293T cells were co-cultured for 12 hours with J774.1 murine macrophage cells which had been stimulated with LPS 12 hours prior. Based on previously reported assays, we used an effector to target cell ratio of 10:1. At this ratio, serum from ΔgD-2::RFP vaccinated mice induced killing of approximately 70.5% of highly infected (RFP^{high}) target cells (FIG. 15B). This was

significantly more than the 49.3% killed in the absence of serum ($p<0.05$). It was investigated whether only RFP^{high} cells would significantly decrease, since RFP in the model is a surrogate for viral protein expression and cells expressing higher levels these proteins will bind more antibodies cross-link FcyRs, initiating ADCK. There was no significant difference in the killing of RFP^{mid} or $RFP^{mid+high}$ (total infected cells) between the two treatment groups (FIG. 15B). Infected target cells were defined as double positive HEK293T cells that expressed RFP in cultures lacking effector cells. RFP^{high} cells were defined as cells expressing RFP above the mean intensity of infected target cells. RFP^{mid} cells were defined as all infected target cells expressing RFP below the mean intensity (FIG. 15A).

[00169] ADCK is significantly increased in the presence of serum from mice vaccinated with ΔgD-2::RFP compared to serum from mice vaccinated with a control cell lysate. Infected HEK 293 cells were incubated with serum from mice vaccinated with either ΔgD-2::RFP or a control VD60 cell lysate to determine whether killing mediated by different immortalized macrophage cells lines was caused by antigen-specific ADCK. For each treatment group, the proportion of RFP^{high} cells after 16 hours of co-culture was compared to that of parallel assays lacking serum. In cultures containing J774.1 macrophages, serum from ΔgD-2::RFP vaccinated mice induced significantly more killing of RFP^{high} cells than serum from VD60 mock-vaccinated mice ($p<0.001$; FIG. 15C). A similar trend was seen in cultures containing Raw 264.7 macrophages ($p=0.09$). Additionally, for both J774.1 and Raw 264.7 macrophages, ADCK measured in cultures containing serum from VD60 mock-vaccinated mice were indistinguishable from cultures lacking serum (mean % difference = -5.9 ± 7.6, -0.3 ± 3.5). This indicates that non-specific antibodies do not induce killing in our assay. These results confirm that the ADCK measured in the RFADCK assay is a result of HSV-specific antibodies elicited by vaccination with ΔgD-2::RFP.

[00170] Tracking of infected cells by live-imaging of ADCK assay shows that cytotoxicity and phagocytosis are not mutually exclusive. ADCK assay was carried out as described above with the exceptions that glass-bottom 96-well plates were used and the effector cells were Raw 264.7 murine macrophage cells which have been shown to carry out similar amounts of ADCC and ADCP. The assays were imaged on a deconvolution microscope at 60x magnification approximately every 15 minutes for 24 hours. Infected cells are RFP^{+}. Uninfected HEK293 cells are highly Mem^{+} and $Live/Dead^{+}$. Macrophages are unstrained. FIG. 15A indicates that different types of cell killing of infected cells (white

arrows) by macrophages are observed at the indicated time points: Macrophages (*) cluster around the left infected cell over time, and the right infected cell undergoes apoptotic blebbing observed at 4.5 hours. These resulting blebs are quickly phagocytosed by macrophages, which fluoresce with RFP observed at 7.5-hours. The controlled apoptosis of this infected cell indicates that ADCC has occurred, but phagocytosis by, and subsequent fluorescence of, macrophages would be measured by flow analysis as ADCP. The total number of infected cells captured was low, leading to high variability (n=58 infected cells/group), but quantification of killing of infected cells across movies from three experiments shows similar mean ADCK in the presence of Raw 264.7 macrophage cells as was measured previously by RFADCK assay ($mean_{RFADCK}$= 18.8%, $mean_{movie}$= 18.5% killing; FIG. 15C, 16B).

[00171] ADCK activity by bone marrow-derived macrophages is FcγR-dependent. RFADCK assays were performed with bone marrow-derived macrophages (BMDMs) from wild-type and $Fc\gamma R^{-/-}$ mice to determine whether observed ADCK was FcγR dependent. ADCK activity was defined as the decrease in RFP^{high} cells compared to parallel cultures with serum from VD60 lysate mock-vaccinated mice. At an effector to target cell ratio of 10:1, wild-type BMDMs killed significantly more infected target cells than $Fc\gamma R^{-/-}$ BMDMs in the presence of serum from HSV-2 ΔgD-2::RFP vaccinated mice ($p<0.05$; FIG. 17A). Additionally, killing of RFP^{high} cells by $Fc\gamma R^{-/-}$ BMDMs in the presence of serum from HSV-2 ΔgD-2::RFP vaccinated mice was indistinguishable from killing in the presence of serum from VD60 lysate mock-vaccinated mice (mean % difference = 3.6 ± 4.0), indicating that ADCK activity measured in our assay is entirely FcγR dependent.

[00172] RFADCK using guinea pig bone marrow-derived macrophages recapitulates murine results. To determine the ability of another species' effector cells to carry out ADCK in our assay, guinea pig BMDMs were co-cultured with ΔgD-2::RFP infected HEK 293 cells in the presence of serum from ΔgD-2 vaccinated, VD60 lysate mock vaccinated, or naïve guinea pigs. The killing of RFP^{high} cells in parallel co-cultures containing serum from naïve animals was used as the baseline. In the presence of serum from ΔgD-2 vaccinated animals, guinea pig BMDMs carried out significant ADCK ($p<0.001$; FIG. 17B).

[00173] Anti-PR8 IgG Isotype ELISA. Mice were subcutaneously injected with ΔgD-2::RFP, ΔgD-2 expressing full-length PR8 hemagglutinin (FL nOP, β3χ4), ΔgD-2 expressing headless PR8 hemagglutinin (HL nOP, β3χ5), or ΔgD-2 expressing headless

PR8 hemagglutinin codon-optimized for HSV-2 (G) (HL OPT, β3χ6) or mock vaccinated with VD60 cell lysate. 5 mice/group were prime-boost vaccinated with each treatment separately. (See FIG. 20). 5 mice were primed with FL nOP and boosted with HL nOP. In each case, $5x10^6$ PFU of virus were given with each injection. At day 30 (9 days post-boost), mice were bled and serum was collected. Enzyme-linked immunosorbent assays (ELISAs) were then run to check for antibodies against purified PR8 HA or Vero cells infected with HSV-2 4674. Mice prime-boosted with FL nOP or primed with FL nOP and boosted with HL nOP developed IgG1 and IgG2c antibodies against PR8 HA. No other group formed anti-PR8 IgG antibodies. All groups except the VD60 lysate group developed similar and high titers of anti-HSV IgGs. Anti-HSV IgGs anti-HSV IgGs were elicited with all the vaccine strains.

Example 4

[00174] Mice vaccinated with ΔgD-2::HA_{PR8} are fully protected from challenge with PR8. Mice were prime-boost vaccinated subcutaneously 3 weeks apart with $5x10^6$ PFU of ΔgD-2::RFP or ΔgD-2::HA_{PR8} or mock vaccinated with VD60 cell lysate. The mice were bled one-week post-boost and serum neutralization titer was measured against A/Puerto Rico/1934/8 IAV (PR8). (See FIG. 21A) Mice immunized with ΔgD-2::HA_{PR8} developed significant neutralizing Ab titers against PR8 (mean = 304). Dotted line represents the limit of detection for the assay. Three weeks post-boost, mice were challenged intranasally with a $6xLD_{50}$ of the PR8 strain. The mice were sacrificed when they reached 75% of their initial weight. As shown in FIGs. 21B and 21C, mice immunized with ΔgD-2::HA_{PR8} were fully protected from PR8 challenge, while mice that received control vaccinations all succumbed to infection before day 9.

[00175] Recombinant gD-2::HA_{PR8} expresses high levels of PR8 protein. To assess expression, ΔgD-2*::RFP* gDNA was co-transfected into VD60 cells alongside an HA expression cassette containing the hemagglutinin (HA) gene from IAV H1N1 strain A/Puerto Rico/1934/8 (PR8) downstream of P_{CMV} and upstream of a poly-adenylation signal as illustrated in FIG. 22A. Extracellular and intracellular HA expression was measured by flow cytometry in Vero and VD60 cells infected with 3 MOI of ΔgD-2, ΔgD-2::HA_{PR8}, or ΔgD-2 containing a truncated version of the PR8 HA expression cassette (ΔgD-2::HL HA_{PR8}), and HA expression is shown in FIG. 22B.

[00176] Mice immunized with gD-2::HA_{PR8} develop high titers of functional and isotype switched anti-PR8 antibodies. Mice were prime-boost vaccinated 21 days apart with either VD60 cell lysate, ΔgD-2::RFP vector, or ΔgD-2::HA_{PR8} (5 mice per group). At day 28 post-prime vaccination, serum was collected for analysis. Anti-PR8 antibodies were measured by ELISA against purified HA PR8 protein. Mice immunized with ΔgD-2::HA_{PR8} developed isotype switch anti-PR8 HA antibodies that were predominantly IgG2c and IgG2b. (See FIGs. 23A-23E) Serum from mice immunized with ΔgD-2::HA_{PR8} induced significant hemagglutination inhibition compared to serum from mice immunized with ΔgD-2 ($p<0.001$; $mean_{HAI\ titer} = 80$). (See FIG. 23F) Mice immunized with ΔgD-2::RFP did not develop any hemagglutination inhibiting antibodies.

[00177] Mice immunized with gD-2::HA_{PR8} develop protection against IAV challenge. Mice were prime-boost immunized 21 days apart with VD60 cell lysate, ΔgD-2, or ΔgD-2*::HA_{PR8}*, bled at day 28 post-prime, and challenged intranasally 14 days later with a 6x LD_{50} of IAV. Mice were sacrificed after reaching 70% of starting weight and neutralization titers were measured using microneutralization assays against the respective strains. As shown in FIGS. 24A-24C, mice immunized with ΔgD-2::HA_{PR8} developed significant neutralizing titers against PR8 (mean $titer_{PR8} = 1:304$; $p_{VD60} < 0.01$; $p_{\Delta gD\text{-}2} < 0.05$; n=10 mice/group). Mice immunized with ΔgD-2::HA_{PR8} were also fully protected from weight loss and mortality followed by challenge with PR8 ($p < 0.001$, n=5 mice/group). As shown in FIGS. 24D-24F, mice immunized with ΔgD-2::HA_{PR8} developed significant neutralizing antibodies against A/California/2009 H1N1 (A/Cal/2009) IAV (mean $titer_{A/Cal} = 26$; $p_{VD60} < 0.01$ $p_{\Delta gD\text{-}2} = 0.1$; n=10 mice/group). Mice immunized with ΔgD*::HA_{PR8}* were partially protected from weight loss and mortality following challenge with A/Cal/2009 ($p < 0.05$, n=15 mice/group). As shown in FIGS. 24G-24I, mice immunized with ΔgD-2*::HA_{PR8}*, ΔgD-2*::RFP*, or VD60 cell lysate did not develop neutralizing antibody titers against A/Victoria/3/75 H3N2 (A/Vic), and A/Aichi/68 H3N2 (X-31) and were not protected from weight loss and mortality following challenge ($n_{neutralization} = 10$ mice/group; $n_{A/Vic}$ challenge = 15 mice/group; $n_{X\text{-}31}$ challenge = 5 mice/group).

[00178] Mice immunized with ΔgD-2::HA_{PR8} develop fully protective ADCC immunity against HSV-2. Mice were prime-boost vaccinated 21 days apart with either VD60 cell lysate, ΔgD-2, or ΔgD-2::HA_{PR8} (5 mice per group). In FIGS. 25A and 25B, the mice were bled 1-week post-boost and the serum was analyzed by ELISA. Mice that received ΔgD-2 or ΔgD-2::HA_{PR8} generated similarly high levels of HSV-specific IgG (FIG. 25A).

Additionally, these IgGs were predominantly IgG2c. In FIG. 25C, rapid fluorometric antibody-dependent cell-mediated killing (RFADCK) assay was carried out using the same serum as FIGs. 25A and 25B. Sera from mice vaccinated with ΔgD-2 or ΔgD-2::HA_{PR8} elicited significant ADCK activity in the presence of J774.1 macrophages and ΔgD-2 infected cells compared with serum from mice given VD60 cell lysate ($p < 0.01$). There was no difference between the ADCK activity elicited by sera from mice vaccinated with ΔgD-2 and ΔgD-2::HA_{PR8}. At 21 days post-boost, mice were challenged by skin scarification with a 10x LD_{90} of HSV-2 4674. Mice that received VD60 cell lysate succumbed to HSV-2 by 10 and developed significant epithelial and neurological disease. Mice that received ΔgD-2 and ΔgD-2::HA_{PR8} were fully protected from morbidity and mortality following challenge. (See FIGS. 25D to 25F)

[00179] mFcγRIV ADCC reporter bioassay response. Serum samples collected from mice vaccinated with ΔgD-2::HA, ΔgD-2::RFP, and inactivated A/Puerto Rico/8/1934 H1N1 virus (PR8) were serially diluted and subjected to mFcγRIV ADCC reporter bioassay. Madin-Darby Canine Kidney (MDCK) cells infected with PR8 virus were used as target cells and mFcγRIV expressing Jurkat T cells were used as effector cells. The target cells were incubated with the serially diluted serum samples and effector cells. Bio-Glo™ Reagent was added, and luminescence was measured. The HSV-2 ΔgD::HA vaccinated mice showed significantly higher activation of mFcγRIV receptors at each dilution tested in comparison to mice vaccinated with ΔgD-2::RFP and inactivated PR8 virus. (See FIG. 26)

Example 5

[00180] Cloning of HIV-1 Env in pBkk412 plasmid containing HSV-2 genes. The full length HIV-1 *Env (Clade C)* and *Rev* genes, together with partial *Nef* gene, were PCR amplified from the plasmid ZM109F.PB4 (FIG. 27) obtained from NIH AIDS Reagent Program.

[00181] The PCR fragment product, which also contained bGH polyA signal and T7 promoter, was cloned into the multiple cloning site (MCS) of the pBkk412 plasmid under control of CMV promoter, using RE *sbf I* and *blp I*. (See FIG. 28)

[00182] Stable, competent E.coli cells were transformed and plated on Agar+ carbenicillin. Colonies were screened for the presence of the insert using PCR (FIG. 29A), and were used to prepare DNA for analysis. The DNA was amplified by PCR and clones

(CL 15-6 and CL 26-11) having the correct size were selected for transient transfection to assess HIV-1 protein expression. (FIG. 29B)

[00183] Once expression of the HIV proteins in transfected cells is confirmed, cells will be co-transfected with the linear recombinant plasmid and the HSV ΔgD-2 negative virus (e.g., ΔgD-2::RFP, ΔgD-2::GFP) and selected for recombinants that express the HIV protein and do not express the GFP or RFP marker. Expression of the HIV protein(s) by the new recombinant virus will be assessed by Western blot or flow cytometry (FACS). Mice will be immunized intramuscularly (prime and boost) and serum collected and screen for the presence of HIV-specific antibodies. The functionality (e.g., neutralizing and non-neutralizing functions) of the Abs will also be measured. A humanized mouse model which renders mice susceptible to infection with HIV, will also be used to assess whether the vaccine protects the mice from HIV infection.

[00184] Set forth below are some embodiments of the process, the recombinant virus, and the methods disclosed herein.

[00185] Embodiment 1: A process for producing a vaccine vector directed against a heterologous antigen, the process comprising: a) providing an HSV-2 genome comprising: (i) a fully or partially deleted in a gene encoding HSV-2 glycoprotein D, and (ii) a nucleic acid comprising a promoter-FP construct, wherein FP is a nucleic acid encoding a fluorescent protein; b) co-transfecting a host cell with (i) the HSV-2 genome of a) and (ii) a linear DNA fragment encoding the heterologous antigen under conditions whereby allelic recombination occurs between the HSV-2 genome and the DNA fragment; c) screening plaques resulting from b) to identify plaques not showing fluorescence under excitation light which elicits fluorescent protein fluorescence; d) recovering from those plaques not showing fluorescence in c) recombinant HSV-2 viruses or virions so as to obtain a vaccine vector directed against the heterologous antigen.

[00186] Embodiment 2: The process of embodiment 1, wherein the host cell is a HSV-1 glycoprotein D complementing cell.

[00187] Embodiment 3: The process of embodiment 1 or embodiment 2, wherein the promoter of the promoter-FP construct is a heterologous promoter.

[00188] Embodiment 4: The process of any of Embodiments 1-3, wherein the co-transfecting is effected by electroporation.

[00189] Embodiment 5: The process of any of Embodiments 1-4, wherein the fluorescent protein is Red Fluorescent Protein (RFP).

[00190] Embodiment 6: The process of any of Embodiments 1-5, wherein the host cell is co-transfected with (i) the HSV-2 genome of a) and (ii) a linear DNA fragment encoding, in order, (i) HSV-2 gD signal sequence, the heterologous antigen, HSV-2 gD transmembrane domain, HSV-2 gD cytosolic domain, but not encoding a HSV-2 gD extracellular domain, or (ii) HSV-2 gD signal sequence, the heterologous antigen, and cytosolic domain of HSV-2 gD.

[00191] Embodiment 7: The process of any of Embodiments 1-5, wherein the host cell is co-transfected with (i) the HSV-2 genome of a) and (ii) a linear DNA fragment encoding, in order, (i) a promoter, the heterologous antigen, and optionally a poly-A signal.

[00192] Embodiment 8: The process of any of Embodiments 1-7, wherein the heterologous antigen is an influenza antigen.

[00193] Embodiment 9: The process of any of Embodiments 1-8, wherein the heterologous antigen is an influenza hemagglutinin (HA) antigen.

[00194] Embodiment 10: The process of Embodiment 9, wherein the HA antigen is a full-length HA extracellular domain or is a HA stalk.

[00195] Embodiment 11: The process of any of Embodiments 1-7, wherein the heterologous antigen is an HIV antigen.

[00196] Embodiment 12: The process of Embodiment 11, wherein the HIV antigen is an Env gp145.

[00197] Embodiment 13: The process of any of Embodiments 1-12, wherein the heterologous antigen is under control of an upstream CMV promoter and has a downstream SV40 poly-A signal.

[00198] Embodiment 14: The process of any of Embodiments 1-13, wherein the promoter is a promoter of Elongation Factor 1α gene ($P_{EF1\alpha}$) and wherein $P_{EF1\alpha}$ and FP are fused together ($P_{EF1\alpha}$-FP).

[00199] Embodiment 15: A vaccine vector, or a recombinant herpes simplex virus-2 (HSV-2) comprising a genome encoding a heterologous antigen, made by the process of any of Embodiments 1-14.

[00200] Embodiment 16: A recombinant herpes simplex virus-2 (HSV-2) comprising: (i) a complete deletion of an HSV-2 glycoprotein D-encoding gene in the genome thereof; and (ii) (a) a linear DNA fragment, encoding a promoter, a heterologous antigen signal sequence, and a heterologous antigen or (b) encoding a promoter, and a heterologous antigen.

[00201] Embodiment 17: A recombinant herpes simplex virus-2 (HSV-2) comprising: (i) a partial deletion of an HSV-2 glycoprotein D-encoding gene in the genome thereof; and (ii) (a) a linear DNA fragment encoding, in order, an HSV-2 gD signal sequence, a heterologous antigen, an HSV-2 gD transmembrane domain, optionally an HSV-2 gD cytosolic domain, but not encoding an HSV-2 gD extracellular domain, or (b) a linear DNA fragment encoding, in order, an HSV-2 gD signal sequence, a heterologous antigen, and transmembrane cytoplasmic tail of HSV-2 gD.

[00202] Embodiment 18: The recombinant HSV-2 of Embodiment 16 or 17, further comprising a parasitic surface glycoprotein on a lipid bilayer thereof, wherein the parasite is a parasite of a mammal.

[00203] Embodiment 19: The recombinant HSV-2 of any of Embodiments 16-18, wherein the HSV-2 glycoprotein D-encoding gene is an HSV-2 *Us6* gene.

[00204] Embodiment 20: The recombinant HSV-2 of any of Embodiments 16-19, wherein the heterologous antigen is an influenza antigen.

[00205] Embodiment 21: The recombinant HSV-2 of any of Embodiments 16-20, wherein the heterologous antigen is hemagglutinin (HA) antigen.

[00206] Embodiment 22: The recombinant HSV-2 of Embodiment 21, wherein the HA antigen is a full-length HA extracellular domain or is a HA stalk.

[00207] Embodiment 23: The recombinant HSV-2 of any of Embodiments 16-19, wherein the heterologous antigen is an HIV antigen.

[00208] Embodiment 24: The recombinant HSV-2 of Embodiment 23, wherein the HIV antigen is an Env gp145.

[00209] Embodiment 25: A cell comprising therein a recombinant virus of any of Embodiments 16-24, wherein the cell is not present in a human being.

[00210] Embodiment 26: A vaccine composition comprising the recombinant virus of any of Embodiments 16-24.

[00211] Embodiment 27: A pharmaceutical composition comprising the virus of any of Embodiments 16-24, and a pharmaceutically acceptable carrier.

[00212] Embodiment 28: A method of eliciting and/or enhancing an immune response in a subject, the method comprising administering to the subject an amount of the recombinant virus of any of Embodiments 16-24, in an amount effective to elicit and/or enhance an immune response in a subject.

[00213] Embodiment 29: A method of eliciting and/or enhancing an immune response in a subject, the method comprising administering to the subject an amount of the vaccine of Embodiment 26 in an amount effective to elicit and/or enhance an immune response in a subject.

[00214] Embodiment 30: A method of eliciting and/or enhancing an immune response in a subject, the method comprising administering to the subject an amount of the pharmaceutical composition of Embodiment 27, in an amount effective to elicit and/or enhance an immune response in a subject.

[00215] Embodiment 31: A method of treating or reducing the likelihood of an influenza infection in a subject, the method comprising administering to the subject an amount of the recombinant virus of any of Embodiments 16-22, in an amount effective to treat or reduce the likelihood of an influenza infection in a subject.

[00216] Embodiment 32: A method of treating or reducing the likelihood of an influenza infection in a subject, the method comprising administering to the subject an amount of the vaccine of Embodiment 26, in an amount effective to treat or reduce the likelihood of an influenza infection in a subject.

[00217] Embodiment 33: A method of treating or reducing the likelihood of an influenza infection in a subject, the method comprising administering to the subject an amount of the pharmaceutical composition of Embodiment 27, in an amount effective to treat or reduce the likelihood of an influenza infection in a subject.

[00218] Embodiment 34: A method of treating or reducing the likelihood of an HIV infection in a subject, the method comprising administering to the subject an amount of the recombinant virus of any of Embodiments 16-19, 23 or 24, in an amount effective to treat or reduce the likelihood of an HIV infection in a subject.

[00219] Embodiment 35: A method of treating or reducing the likelihood of an HIV infection in a subject, the method comprising administering to the subject an amount of the vaccine of Embodiment 26, in an amount effective to treat or reduce the likelihood of an HIV infection in a subject.

[00220] Embodiment 36: A method of treating or reducing the likelihood of an HIV infection in a subject, the method comprising administering to the subject an amount of the pharmaceutical composition of Embodiment 27, in an amount effective to treat or reduce the likelihood of an HIV infection in a subject.

[00221] Embodiment 37: A method of vaccinating a subject for influenza infection, the method comprising administering to the subject an amount of the recombinant virus of any of Embodiments 16-22, in an amount effective to vaccinate a subject for influenza infection.

[00222] Embodiment 38: A method of vaccinating a subject for influenza infection, the method comprising administering to the subject an amount of the vaccine of Embodiment 26, in an amount effective to vaccinate a subject for influenza infection.

[00223] Embodiment 39: A method of vaccinating a subject for influenza infection, the method comprising administering to the subject an amount of the pharmaceutical composition of Embodiment 27, in an amount effective to vaccinate a subject for influenza infection.

[00224] Embodiment 40: A method of vaccinating a subject for HIV infection, the method comprising administering to the subject an amount of the recombinant virus of any of Embodiments 16-19, 23 or 24, in an amount effective to vaccinate a subject for HIV infection.

[00225] Embodiment 41: A method of vaccinating a subject for HIV infection, the method comprising administering to the subject an amount of the vaccine of Embodiment 26, in an amount effective to vaccinate a subject for HIV infection.

[00226] Embodiment 42: A method of vaccinating a subject for HIV infection, the method comprising administering to the subject an amount of the pharmaceutical composition of Embodiment 27, in an amount effective to vaccinate a subject for HIV infection.

[00227] Embodiment 43: A method of eliciting and/or enhancing an immune response in a subject, the method comprising administering to the subject an amount of a recombinant herpes simplex virus-2 (HSV-2) made by the process of any of Embodiments 1-14 and comprising (i) a complete deletion of an HSV-2 glycoprotein D-encoding gene in the genome thereof and (ii) encoding a promoter, an influenza hemagglutinin (HA) antigen signal sequence, and an HA antigen in an amount effective to elicit and/or enhance an immune response in a subject.

[00228] Embodiment 44: A method of treating or reducing the likelihood of an influenza infection in a subject, the method comprising administering to the subject an amount of a recombinant herpes simplex virus-2 (HSV-2) made by the process of any of Embodiments 1-13 and comprising (i) a complete deletion of an HSV-2 glycoprotein D-encoding gene in the genome thereof and (ii) encoding a promoter, an influenza hemagglutinin (HA) antigen

signal sequence, and an HA antigen in an amount effective to treat or reduce the likelihood of an influenza infection in a subject.

[00229] Embodiment 45: A method of vaccinating a subject for influenza infection, the method comprising administering to the subject an amount of a recombinant herpes simplex virus-2 made by the process of any of Embodiments 1-14 and comprising (i) a complete deletion of an HSV-2 glycoprotein D-encoding gene in the genome thereof and (ii) encoding a promoter, an influenza hemagglutinin (HA) antigen signal sequence, and an HA antigen in an amount effective to vaccinate a subject for influenza infection.

[00230] Embodiment 46: The method of any of Embodiments 43-45, wherein the HA antigen is a full-length HA extracellular domain.

[00231] Embodiment 47: The method of Embodiment 46, further comprising, subsequent to an initial administration of the recombinant herpes simplex virus-2 encoding a full-length HA extracellular domain, administering one or more amounts of a recombinant herpes simplex virus-2 comprising (i) a complete deletion of an HSV-2 glycoprotein D-encoding gene in the genome thereof and (ii) encoding a promoter, a HA antigen signal sequence, and an HA stalk, but not encoding a full-length HA.

[00232] Embodiment 48: The method of any of Embodiments 28-47, wherein the subject is a human being.

[00233] Embodiment 49: A method of quantitating a rate or amount of antibody-dependent cell-mediated killing (ADCK) in a population of cells, the method comprising: infecting a plurality of cells of the population of cells with a fluorescent protein-expressing recombinant HSV-2 that comprises a genome deleted for the gene encoding HSV-2 gD, under conditions permitting expression of the fluorescent protein in the cells, contacting the plurality of infected cells with an antibody-containing solution and a population of immune cells, and quantitating at one or more time points the amount of the plurality of infected cells exhibiting fluorescent protein fluorescence and, optionally, one or more markers, so as to quantitate over time the amount of live infected cells, so as to thereby quantitating the rate or amount of ADCK in the population of cells.

[00234] Embodiment 50: The method of Embodiment 49, wherein the method is performed *in vitro*.

[00235] Embodiment 51: The method of Embodiment 49 or 50, wherein the population of immune cells comprises macrophages.

[00236] Embodiment 52: The method of any of Embodiments 49-51, wherein the antibody-containing solution comprises serum.

[00237] Embodiment 53: The method of any of Embodiments 49-51, wherein the fluorescent protein is Red Fluorescent Protein.

[00238] Embodiment 54: The method of any of Embodiments 49-53, wherein the amount of the plurality of infected cells exhibiting fluorescent protein fluorescence and, optionally, one or more markers, is measured by fluorescence-activated cell sorting (FACS).

[00239] Embodiment 55: The method of any of Embodiments 49-55, wherein the at least one marker comprises a cell membrane marker, a live/dead marker, or a combination thereof.

[00240] Embodiment 56: The method of any of Embodiments 49-55, further comprising quantitating at one or more time points the amount of cells exhibiting fluorescent protein fluorescence and, optionally, one or more markers, in a control population of infected cells otherwise identical but not contacted with an antibody-containing solution and comparing the amount or rate quantitated to the amount or rate quantitated for the population of cells contacted with the antibody-containing solution.

[00241] Embodiment 57: The method of any of Embodiments 49-56, wherein the population of immune cells is present at effector:target ratio of 5:1 or greater.

[00242] Embodiment 58: The method of any of Embodiments 49-57, wherein the recombinant HSV-2 is made by the process of any of Embodiments 1-14.

[00243] Throughout this application various publications are referred to. Full citations for these references may be found at the end of the specification. The disclosures of these publications, and all patents, patent application publications and books referred to herein, are hereby incorporated by reference in their entirety into the subject application to more fully describe the art to which the subject invention pertains.

[00244] One skilled in the art will readily appreciate that the specific methods and results discussed hereinabove are merely illustrative of the invention as described more fully in the claims that follow thereafter.

REFERENCES

Bouvier, N. M. and A. C. Lowen (2010). "Animal Models for Influenza Virus Pathogenesis and Transmission." Viruses 2(8): 1530-1563.

Cheshenko, N., J. B. Trepanier, M. Stefanidou, N. Buckley, P. Gonzalez, W. Jacobs and B. C. Herold (2013). "HSV activates Akt to trigger calcium release and promote viral entry: novel candidate target for treatment and suppression." FASEB J 27(7): 2584-2599.

Eisenberg, R. J., D. Long, R. Hogue-Angeletti and G. H. Cohen (1984). "Amino-terminal sequence of glycoprotein D of herpes simplex virus types 1 and 2." J Virol 49(1): 265-268.

Gibson, D. G., L. Young, R. Y. Chuang, J. C. Venter, C. A. Hutchison, 3rd and H. O. Smith (2009). "Enzymatic assembly of DNA molecules up to several hundred kilobases." Nat Methods 6(5): 343-345.

Haynes, B. F., P. B. Gilbert, M. J. McElrath, S. Zolla-Pazner, G. D. Tomaras, S. M. Alam, D. T. Evans, D. C. Montefiori, C. Karnasuta, R. Sutthent, H. X. Liao, A. L. DeVico, G. K. Lewis, C. Williams, A. Pinter, Y. Fong, H. Janes, A. DeCamp, Y. Huang, M. Rao, E. Billings, N. Karasavvas, M. L. Robb, V. Ngauy, M. S. de Souza, R. Paris, G. Ferrari, R. T. Bailer, K. A. Soderberg, C. Andrews, P. W. Berman, N. Frahm, S. C. De Rosa, M. D. Alpert, N. L. Yates, X. Shen, R. A. Koup, P. Pitisuttithum, J. Kaewkungwal, S. Nitayaphan, S. Rerks-Ngarm, N. L. Michael and J. H. Kim (2012). "Immune-correlates analysis of an HIV-1 vaccine efficacy trial." N Engl J Med 366(14): 1275-1286.

He, W., G. S. Tan, C. E. Mullarkey, A. J. Lee, M. M. Lam, F. Krammer, C. Henry, P. C. Wilson, A. A. Ashkar, P. Palese and M. S. Miller (2016). "Epitope specificity plays a critical role in regulating antibody-dependent cell-mediated cytotoxicity against influenza A virus." Proc Natl Acad Sci U S A 113(42): 11931-11936.

Jegaskanda S., P. C. R., and Stephen J. Kent (2014). "Influenza-Specific Antibody-Dependent Cellular Cytotoxicity: Toward a Universal Influenza Vaccine." The Journal of Immunology.

Liao, H. X., R. Lynch, T. Zhou, F. Gao, S. M. Alam, S. D. Boyd, A. Z. Fire, K. M. Roskin, C. A. Schramm, Z. Zhang, J. Zhu, L. Shapiro, N. C. S. Program, J. C. Mullikin, S. Gnanakaran, P. Hraber, K. Wiehe, G. Kelsoe, G. Yang, S. M. Xia, D. C. Montefiori, R. Parks, K. E. Lloyd, R. M. Scearce, K. A. Soderberg, M. Cohen, G. Kamanga, M. K. Louder,

L. M. Tran, Y. Chen, F. Cai, S. Chen, S. Moquin, X. Du, M. G. Joyce, S. Srivatsan, B. Zhang, A. Zheng, G. M. Shaw, B. H. Hahn, T. B. Kepler, B. T. Korber, P. D. Kwong, J. R. Mascola and B. F. Haynes (2013). "Co-evolution of a broadly neutralizing HIV-1 antibody and founder virus." Nature 496(7446): 469-476.

Mullarkey, C. E., M. J. Bailey, D. A. Golubeva, G. S. Tan, R. Nachbagauer, W. He, K. E. Novakowski, D. M. Bowdish, M. S. Miller and P. Palese (2016). "Broadly Neutralizing Hemagglutinin Stalk-Specific Antibodies Induce Potent Phagocytosis of Immune Complexes by Neutrophils in an Fc-Dependent Manner." MBio 7(5).

Nicola, A. V., S. H. Willis, N. N. Naidoo, R. J. Eisenberg and G. H. Cohen (1996). "Structure-function analysis of soluble forms of herpes simplex virus glycoprotein D." J Virol 70(6): 3815-3822.

Osterholm, M. T., N. S. Kelley, A. Sommer and E. A. Belongia (2012). "Efficacy and effectiveness of influenza vaccines: a systematic review and meta-analysis." Lancet Infect Dis 12(1): 36-44.

Petro, C., P. A. Gonzalez, N. Cheshenko, T. Jandl, N. Khajoueinejad, A. Benard, M. Sengupta, B. C. Herold and W. R. Jacobs (2015). "Herpes simplex type 2 virus deleted in glycoprotein D protects against vaginal, skin and neural disease." Elife 4.

Petro, C. D., B. Weinrick, N. Khajoueinejad, C. Burn, R. Sellers, W. R. Jacobs, Jr. and B. C. Herold (2016). "HSV-2 DeltagD elicits FcgammaR-effector antibodies that protect against clinical isolates." JCI Insight 1(12).

Wang, B. Z., W. Liu, S. M. Kang, M. Alam, C. Huang, L. Ye, Y. Sun, Y. Li, D. L. Kothe, P. Pushko, T. Dokland, B. F. Haynes, G. Smith, B. H. Hahn and R. W. Compans (2007). "Incorporation of high levels of chimeric human immunodeficiency virus envelope glycoproteins into virus-like particles." J Virol 81(20): 10869-10878.

Wohlbold, T. J., R. Nachbagauer, I. Margine, G. S. Tan, A. Hirsh and F. Krammer (2015). "Vaccination with soluble headless hemagglutinin protects mice from challenge with divergent influenza viruses." Vaccine 33(29): 3314-3321.

What is claimed is:

1. A process for producing a vaccine vector directed against a heterologous antigen, the process comprising:

a) providing an HSV-2 genome comprising:

(i) a fully or partially deleted in a gene encoding HSV-2 glycoprotein D, and

(ii) a nucleic acid comprising a promoter-FP construct, wherein FP is a nucleic acid encoding a fluorescent protein;

b) co-transfecting a host cell with (i) the HSV-2 genome of a) and (ii) a linear DNA fragment encoding the heterologous antigen under conditions whereby allelic recombination occurs between the HSV-2 genome and the DNA fragment;

c) screening plaques resulting from b) to identify plaques not showing fluorescence under excitation light which elicits fluorescent protein fluorescence; and

d) recovering from those plaques not showing fluorescence in c) recombinant HSV-2 viruses or virions so as to obtain a vaccine vector directed against the heterologous antigen.

2. The process of Claim 1, wherein the host cell is a HSV-1 glycoprotein D complementing cell.

3. The process of Claim 1 or Claim 2, wherein the promoter of the promoter-FP construct is a heterologous promoter.

4. The process of any of Claims 1-3, wherein the co-transfecting is effected by electroporation.

5. The process of any of Claims 1-4, wherein the fluorescent protein is Red Fluorescent Protein (RFP).

6. The process of any of Claims 1-5, wherein the host cell is co-transfected with (i) the HSV-2 genome of a) and (ii) a linear DNA fragment encoding, in order, (i) HSV-2 gD signal sequence, the heterologous antigen, HSV-2 gD transmembrane domain, HSV-2 gD cytosolic domain, but not encoding a HSV-2 gD extracellular domain, or (ii) HSV-2 gD signal sequence, the heterologous antigen, and cytosolic domain of HSV-2 gD.

7. The process of any of Claims 1-5, wherein the host cell is co-transfected with (i) the HSV-2 genome of a) and (ii) a linear DNA fragment encoding, in order, (i) a promoter, the heterologous antigen, and optionally a poly-A signal.

8. The process of any of Claims 1-7, wherein the heterologous antigen is an influenza antigen.

9. The process of any of Claims 1-8, wherein the heterologous antigen is an influenza hemagglutinin (HA) antigen.

10. The process of Claim 9, wherein the HA antigen is a full-length HA extracellular domain or is a HA stalk.

11. The process of any of Claims 1-7, wherein the heterologous antigen is an HIV antigen.

12. The process of Claim 11, wherein the HIV antigen is an Env gp145.

13. The process of any of Claims 1-12, wherein the heterologous antigen is under control of an upstream CMV promoter and has a downstream SV40 poly-A signal.

14. The process of any of Claims 1-13, wherein the promoter is a promoter of Elongation Factor 1α gene ($P_{EF1\alpha}$) and wherein $P_{EF1\alpha}$ and FP are fused together ($P_{EF1\alpha}$-FP).

15. A vaccine vector, or a recombinant herpes simplex virus-2 (HSV-2) comprising a genome encoding a heterologous antigen, made by the process of any of Claims 1-14.

16. A recombinant herpes simplex virus-2 (HSV-2) comprising:

(i) a complete deletion of an HSV-2 glycoprotein D-encoding gene in the genome thereof; and

(ii) (a) a linear DNA fragment encoding a promoter, a heterologous antigen signal sequence, and a heterologous antigen or (b) encoding a promoter, and a heterologous antigen.

17. A recombinant herpes simplex virus-2 (HSV-2) comprising:

(i) a partial deletion of an HSV-2 glycoprotein D-encoding gene in the genome thereof; and

(ii) (a) a linear DNA fragment encoding, in order, an HSV-2 gD signal sequence, a heterologous antigen, an HSV-2 gD transmembrane domain, optionally an HSV-2 gD cytosolic domain, but not encoding an HSV-2 gD extracellular domain, or (b) a linear DNA fragment encoding, in order, an HSV-2 gD signal sequence, a heterologous antigen, and transmembrane cytoplasmic tail of HSV-2 gD.

18. The recombinant HSV-2 of Claim 16 or 17, further comprising a parasitic surface glycoprotein on a lipid bilayer thereof, wherein the parasite is a parasite of a mammal.

19. The recombinant HSV-2 of any of Claims 16-18, wherein the HSV-2 glycoprotein D-encoding gene is an HSV-2 *Us6* gene.

20. The recombinant HSV-2 of any of Claims 16-19, wherein the heterologous antigen is an influenza antigen.

21. The recombinant HSV-2 of any of Claims 16-20, wherein the heterologous antigen is hemagglutinin (HA) antigen.

22. The recombinant HSV-2 of Claim 21, wherein the HA antigen is a full-length HA extracellular domain or is a HA stalk.

23. The recombinant HSV-2 of any of Claims 16-19, wherein the heterologous antigen is an HIV antigen.

24. The recombinant HSV-2 of Claim 23, wherein the HIV antigen is an Env gp145.

25. A cell comprising therein a recombinant virus of any of Claims 16-24, wherein the cell is not present in a human being.

26. A vaccine composition comprising the recombinant virus of any of Claims 16-24.

27. A pharmaceutical composition comprising the virus of any of Claims 16-24, and a pharmaceutically acceptable carrier.

28. A method of eliciting and/or enhancing an immune response in a subject, the method comprising administering to the subject an amount of the recombinant virus of any of Claims 16-24, in an amount effective to elicit and/or enhance an immune response in a subject.

29. A method of eliciting and/or enhancing an immune response in a subject, the method comprising administering to the subject an amount of the vaccine of Claim 26 in an amount effective to elicit and/or enhance an immune response in a subject.

30. A method of eliciting and/or enhancing an immune response in a subject, the method comprising administering to the subject an amount of the pharmaceutical composition of Claim 27, in an amount effective to elicit and/or enhance an immune response in a subject.

31. A method of treating or reducing the likelihood of an influenza infection in a subject, the method comprising administering to the subject an amount of the recombinant virus of any of Claims 16-22, in an amount effective to treat or reduce the likelihood of an influenza infection in a subject.

32. A method of treating or reducing the likelihood of an influenza infection in a subject, the method comprising administering to the subject an amount of the vaccine of Claim 26, in an amount effective to treat or reduce the likelihood of an influenza infection in a subject.

33. A method of treating or reducing the likelihood of an influenza infection in a subject, the method comprising administering to the subject an amount of the pharmaceutical composition of Claim 27, in an amount effective to treat or reduce the likelihood of an influenza infection in a subject.

34. A method of treating or reducing the likelihood of an HIV infection in a subject, the method comprising administering to the subject an amount of the recombinant virus of any of Claims 16-19, 23 or 24, in an amount effective to treat or reduce the likelihood of an HIV infection in a subject.

35. A method of treating or reducing the likelihood of an HIV infection in a subject, the method comprising administering to the subject an amount of the vaccine of Claim 26, in an amount effective to treat or reduce the likelihood of an HIV infection in a subject.

36. A method of treating or reducing the likelihood of an HIV infection in a subject, the method comprising administering to the subject an amount of the pharmaceutical composition of Claim 27, in an amount effective to treat or reduce the likelihood of an HIV infection in a subject.

37. A method of vaccinating a subject for influenza infection, the method comprising administering to the subject an amount of the recombinant virus of any of Claims 16-22, in an amount effective to vaccinate a subject for influenza infection.

38. A method of vaccinating a subject for influenza infection, the method comprising administering to the subject an amount of the vaccine of Claim 26, in an amount effective to vaccinate a subject for influenza infection.

39. A method of vaccinating a subject for influenza infection, the method comprising administering to the subject an amount of the pharmaceutical composition of Claim 27, in an amount effective to vaccinate a subject for influenza infection.

40. A method of vaccinating a subject for HIV infection, the method comprising administering to the subject an amount of the recombinant virus of any of Claims 16-19, 23 or 24, in an amount effective to vaccinate a subject for HIV infection.

41. A method of vaccinating a subject for HIV infection, the method comprising administering to the subject an amount of the vaccine of Claim 26, in an amount effective to vaccinate a subject for HIV infection.

42. A method of vaccinating a subject for HIV infection, the method comprising administering to the subject an amount of the pharmaceutical composition of Claim 27, in an amount effective to vaccinate a subject for HIV infection.

43. A method of eliciting and/or enhancing an immune response in a subject, the method comprising administering to the subject an amount of a recombinant herpes simplex virus-2 (HSV-2) made by the process of any of Claims 1-14 and comprising (i) a complete deletion of an HSV-2 glycoprotein D-encoding gene in the genome thereof and (ii) encoding a promoter, a influenza hemagglutinin (HA) antigen signal sequence, and an HA antigen in an amount effective to elicit and/or enhance an immune response in a subject.

44. A method of treating or reducing the likelihood of an influenza infection in a subject, the method comprising administering to the subject an amount of a recombinant herpes simplex virus-2 (HSV-2) made by the process of any of Claims 1-13 and comprising (i) a complete deletion of an HSV-2 glycoprotein D-encoding gene in the genome thereof and (ii) encoding a promoter, a influenza hemagglutinin (HA) antigen signal sequence, and an HA antigen in an amount effective to treat or reduce the likelihood of an influenza infection in a subject.

45. A method of vaccinating a subject for influenza infection, the method comprising administering to the subject an amount of a recombinant herpes simplex virus-2 made by the process of any of Claims 1-14 and comprising (i) a complete deletion of an HSV-2 glycoprotein D-encoding gene in the genome thereof and (ii) encoding a promoter, a influenza hemagglutinin (HA) antigen signal sequence, and an HA antigen in an amount effective to vaccinate a subject for influenza infection.

46. The method of any of Claims 43-45, wherein the HA antigen is a full-length HA extracellular domain.

47. The method of Claim 46, further comprising, subsequent to an initial administration of the recombinant herpes simplex virus-2 encoding a full-length HA extracellular domain, administering one or more amounts of a recombinant herpes simplex virus-2 comprising (i) a complete deletion of an HSV-2 glycoprotein D-encoding gene in the genome thereof and (ii) encoding a promoter, a HA antigen signal sequence, and an HA stalk, but not encoding a full-length HA.

48. The method of any of Claims 28-47, wherein the subject is a human being.

49. A method of quantitating a rate or amount of antibody-dependent cell-mediated killing (ADCK) in a population of cells, the method comprising:

infecting a plurality of cells of the population of cells with a fluorescent protein-expressing recombinant HSV-2 that comprises a genome deleted for the gene encoding HSV-2 gD, under conditions permitting expression of the fluorescent protein in the cells,

contacting the plurality of infected cells with an antibody-containing solution and a population of immune cells, and

quantitating at one or more time points the amount of the plurality of infected cells exhibiting fluorescent protein fluorescence and, optionally, one or more markers, so as to quantitate over time the amount of live infected cells, so as to thereby quantitating the rate or amount of ADCK in the population of cells.

50. The method of Claim 49, wherein the method is performed *in vitro.*

51. The method of Claim 49 or 50, wherein the population of immune cells comprises macrophages.

52. The method of any of Claims 49-51, wherein the antibody-containing solution comprises serum.

53. The method of any of Claims 49-51, wherein the fluorescent protein is Red Fluorescent Protein.

54. The method of any of Claims 49-53, wherein the amount of the plurality of infected cells exhibiting fluorescent protein fluorescence and, optionally, one or more markers, is measured by fluorescence-activated cell sorting (FACS).

55. The method of any of Claims 49-55, wherein the at least one marker comprises a cell membrane marker, a live/dead marker, or a combination thereof.

56. The method of any of Claims 49-55, further comprising quantitating at one or more time points the amount of cells exhibiting fluorescent protein fluorescence and, optionally, one or more markers, in a control population of infected cells otherwise identical but not contacted with an antibody-containing solution and comparing the amount or rate quantitated to the amount or rate quantitated for the population of cells contacted with the antibody-containing solution.

57. The method of any of Claims 49-56, wherein the population of immune cells is present at effector:target ratio of 5:1 or greater.

58. The method of any of Claims 49-57, wherein the recombinant HSV-2 is made by the process of any of Claims 1-14.

FIG. 1A

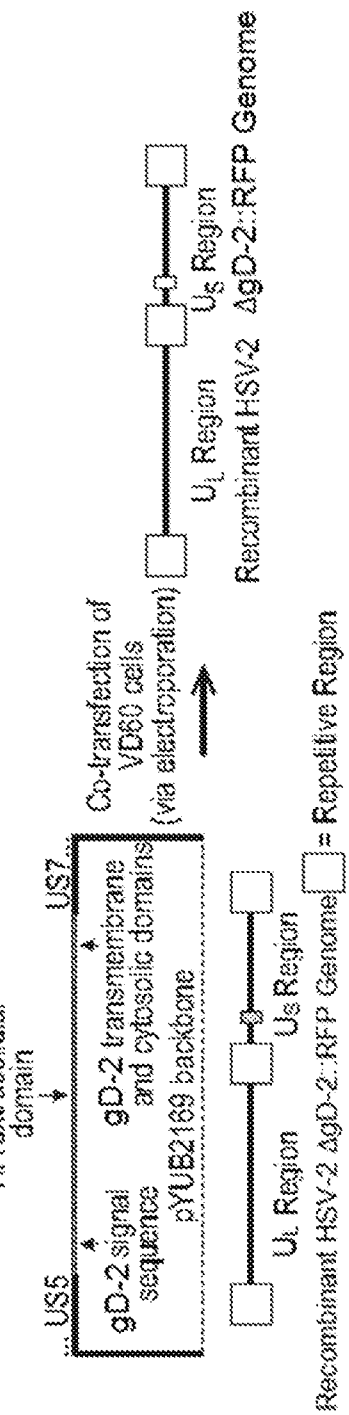
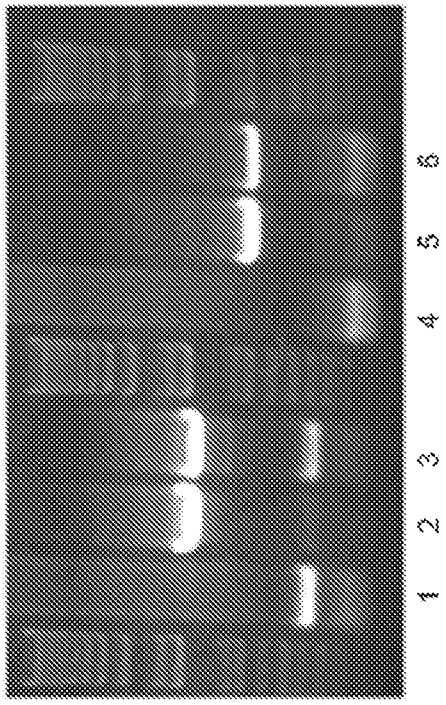

HA extracellular domain
US5
US7
gD-2 signal sequence
gD-2 transmembrane and cytosolic domains
pYUB2169 backbone
Co-transfection of VD60 cells (via electroporation)
U_L Region
U_S Region
Recombinant HSV-2 ΔgD-2::RFP Genome
U_L Region
U_S Region
Recombinant HSV-2 ΔgD-2::RFP Genome
= Repetitive Region

FIG. 1B


1
2
3
4
5
6

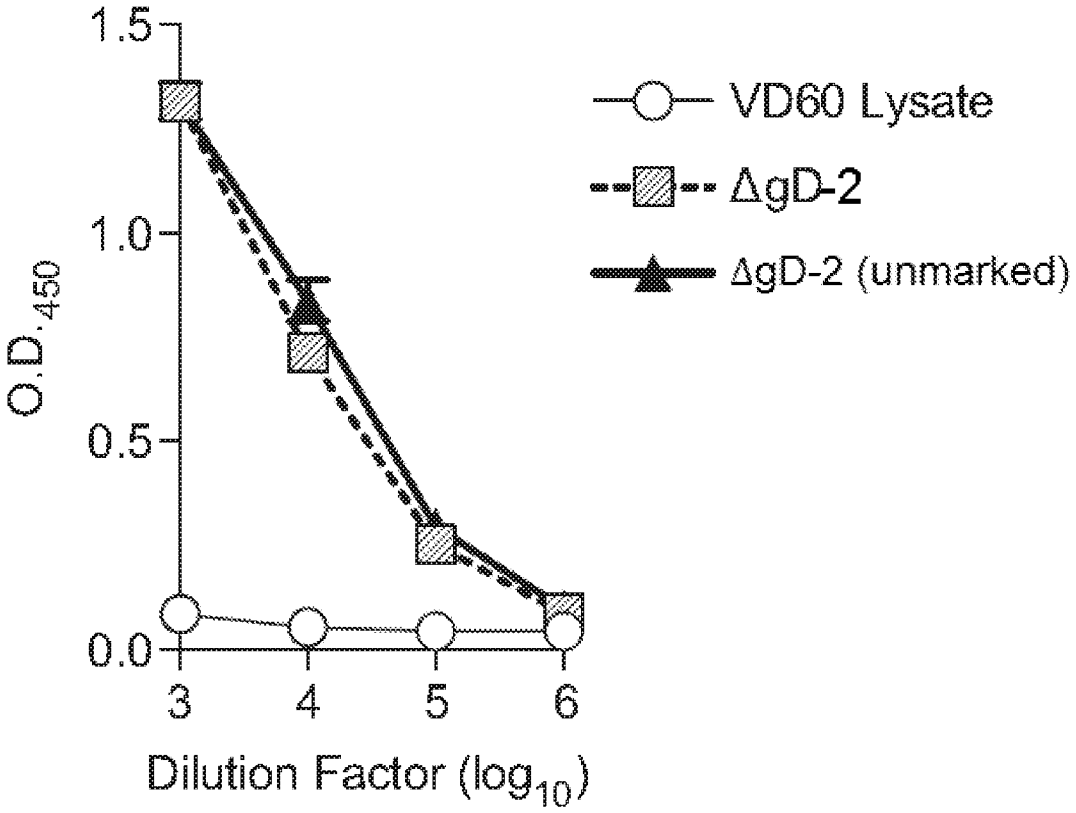

1.5
1.0
0.5
0.0
O.D.450
3
4
5
6
Dilution Factor (log10)
VD60 Lysate
ΔgD-2
ΔgD-2 (unmarked)

FIG. 2

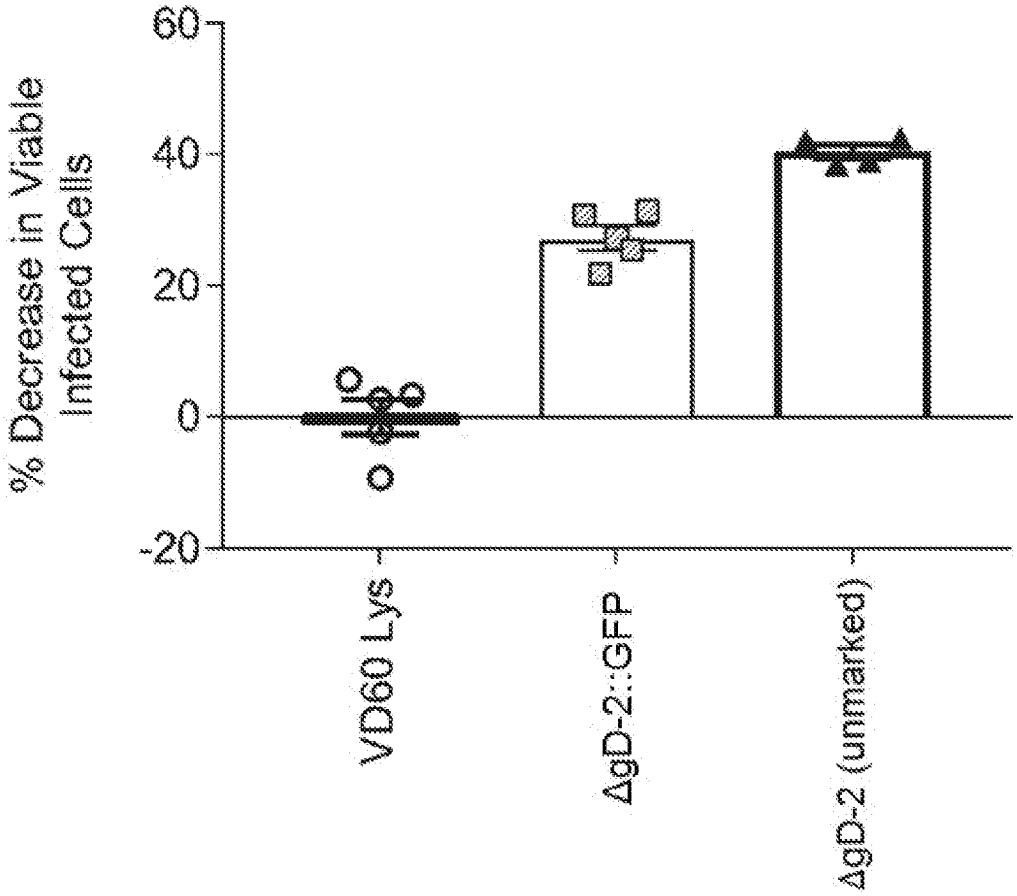

% Decrease in Viable Infected Cells
60
40
20
0
-20
VD60 Lys
ΔgD-2::GFP
ΔgD-2 (unmarked)

FIG. 3

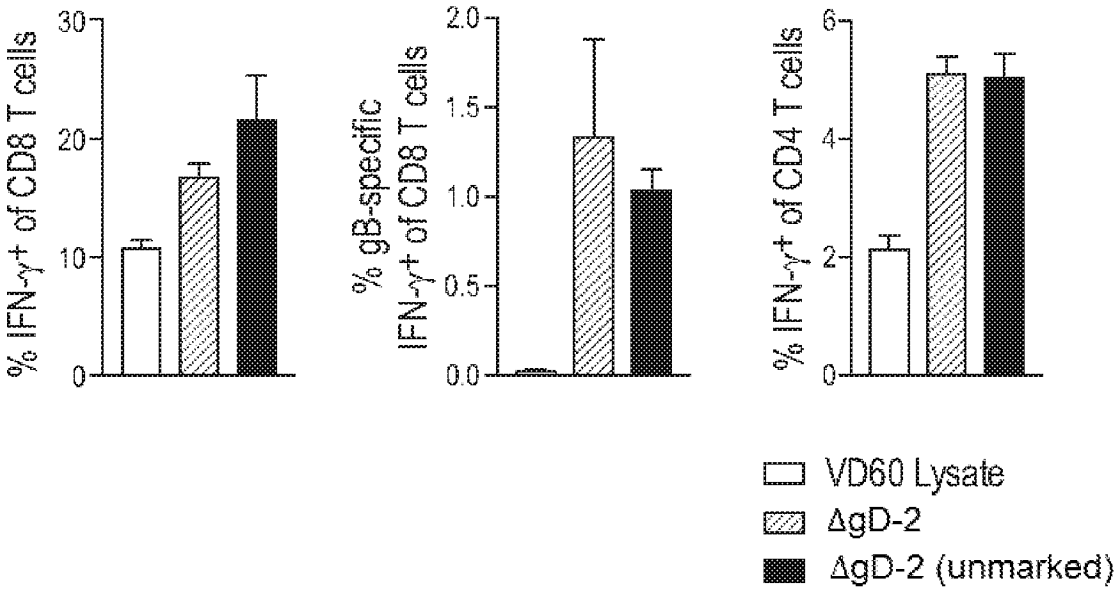

% IFN-γ+ of CD8 T cells
30
20
10
0
% gB-specific IFN-γ+ of CD8 T cells
2.0
1.5
1.0
0.5
0.0
% IFN-γ+ of CD4 T cells
6
4
2
0
VD60 Lysate
ΔgD-2
ΔgD-2 (unmarked)

FIG. 4

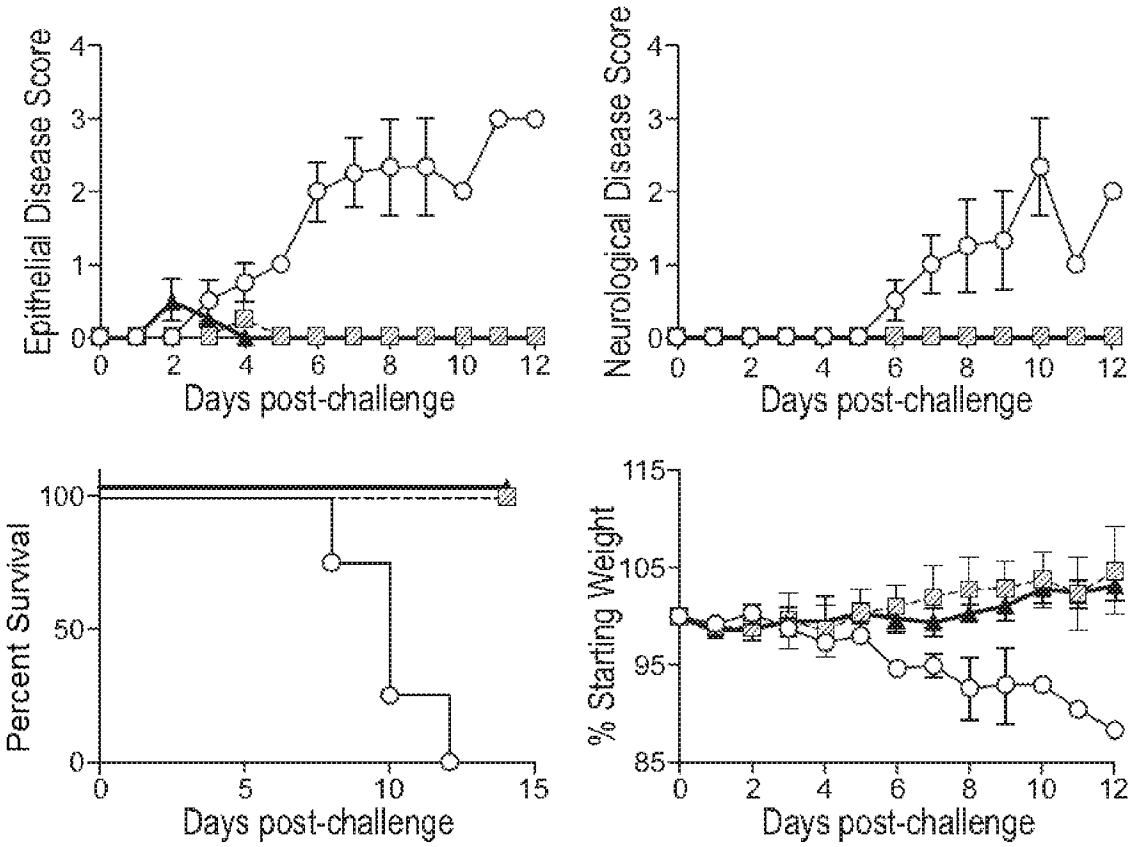

Epithelial Disease Score
Days post-challenge
Neurological Disease Score
Days post-challenge
Percent Survival
Days post-challenge
% Starting Weight
Days post-challenge
VD60 Lysate
ΔgD-2::GFP
ΔgD-2

FIG. 5

SEQ ID NO. 2

MKANLLVLLCALAAADADTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLEDSHNG

KLCRLKGIAPLQLGKCNIAGWLLGNPECDPLLPVRSWSYIVETPNSENGICYPGDFIDYEE

LREQLSSVSSFERFEIFPKESSWPNHNTTKGVTAACSHAGKSSFYRNLLWLTEKEGSYPK

LKNSYVNKKGKEVLVLWGIHHPSNSKDQQNIYQNENAYVSVVTSNYNRRFTPEIAERP

KVRDQAGRMNYYWTLLKPGDTIIFEANGNLIAPRYAFALSRGFGSGIITSNASMHECN

TKCQTPLGAINSSLPFQNIHPVTIGECPKYVRSAKLRMVTGLRNIPSIQSRGLFGAIAGFI

EGGWTGMIDGWYGYHHQNEQGSGYAADQKSTQNAINGITNKVNSVIEKMNIQFTAVG

KEFNKLEKRMENLNKKVDDGFLDIWTYNAELLVLLENERTLDFHDSNVKNLYEKVKSQ

LKNNAKEIGNGCFEFYHKCDNECMESVRNGTYDYPKYSEESKLNREKVDGVKLESM

GIYQILAIYSTVASSLVLLVSLGAISFWMCSNGSLQCRICI*D*AIFRNAMRKNTLVSF

Green = HA1 Stalk

Gray = HA1 Head

Yellow = HA2 Stalk

Blue = HA2 Transmembrane

Pink = HA2 Cytosolic

~~Strikethrough~~ = Genetic material after stop codon

Underline = Fusion peptide

Bold and Italic = Disulfide bond sites

O = Glycosylation Site for PR8 Virus

Glycosylation sites vary from virus to virus

FIG. 6

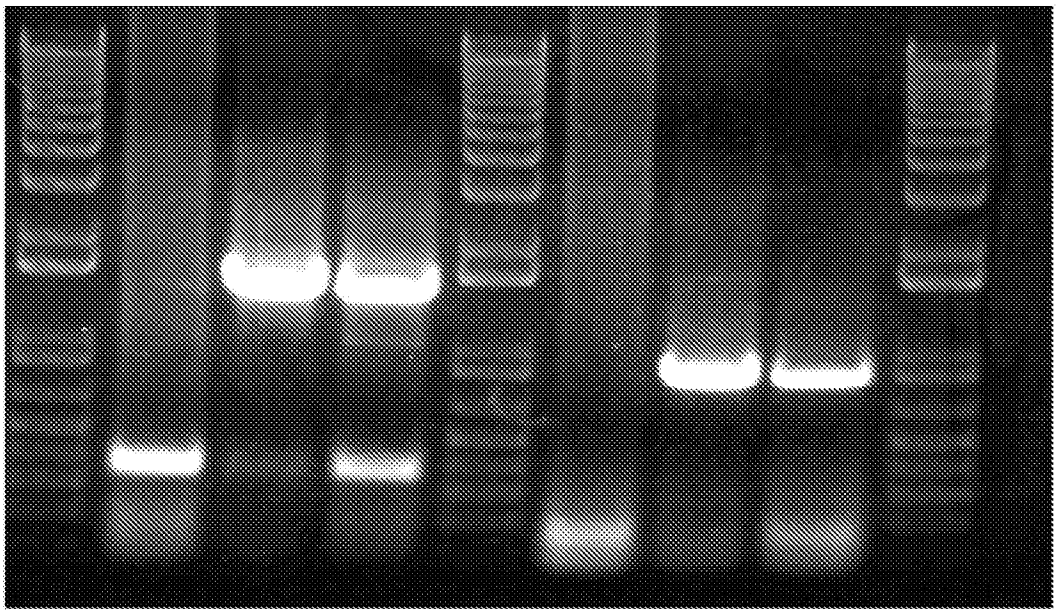


FIG. 7

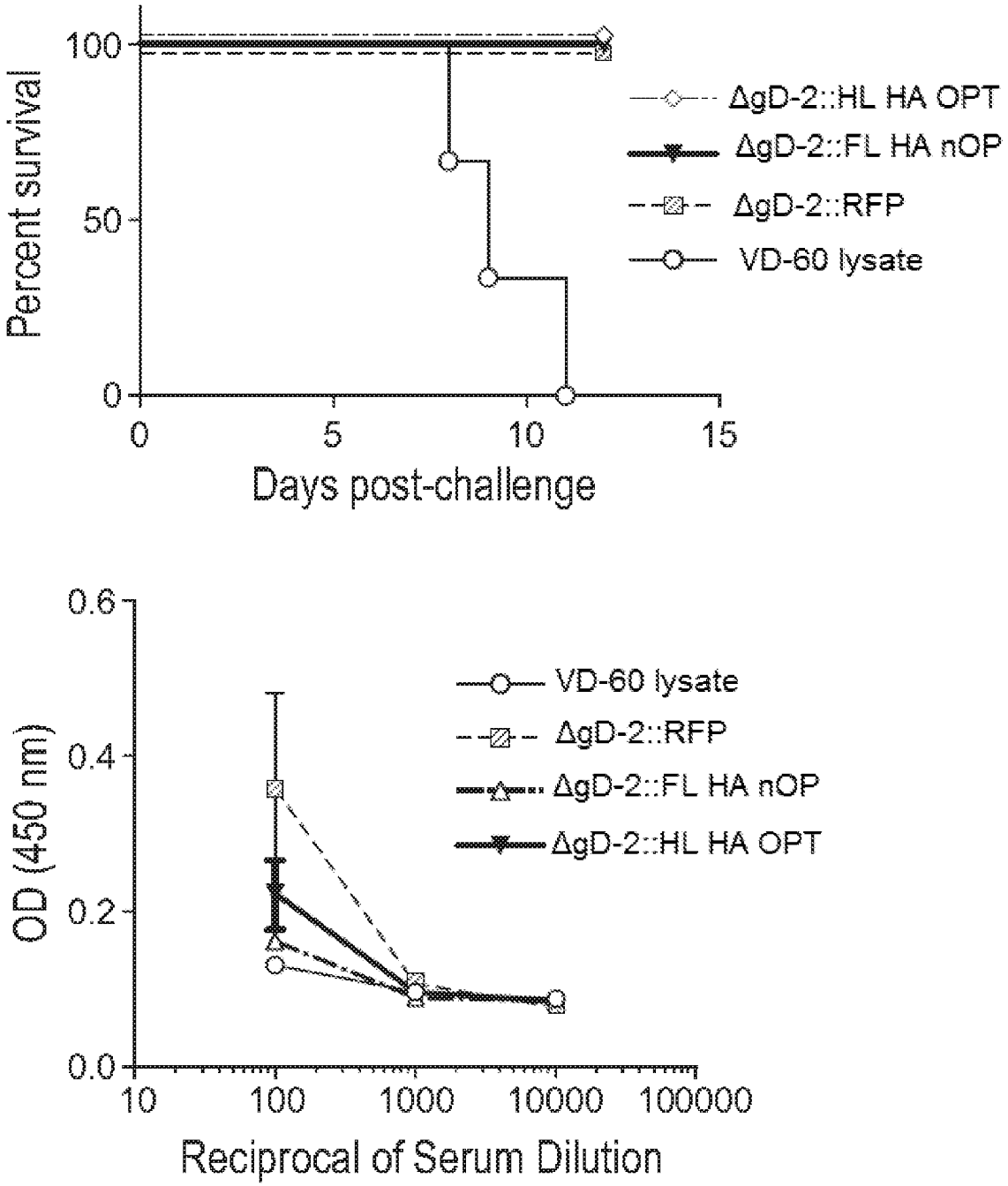

Percent survival
100
50
0
0
5
10
15
Days post-challenge
ΔgD-2::HL HA OPT
ΔgD-2::FL HA nOP
ΔgD-2::RFP
VD-60 lysate
OD (450 nm)
0.6
0.4
0.2
0.0
10
100
1000
10000
100000
Reciprocal of Serum Dilution
VD-60 lysate
ΔgD-2::RFP
ΔgD-2::FL HA nOP
ΔgD-2::HL HA OPT

FIG. 8

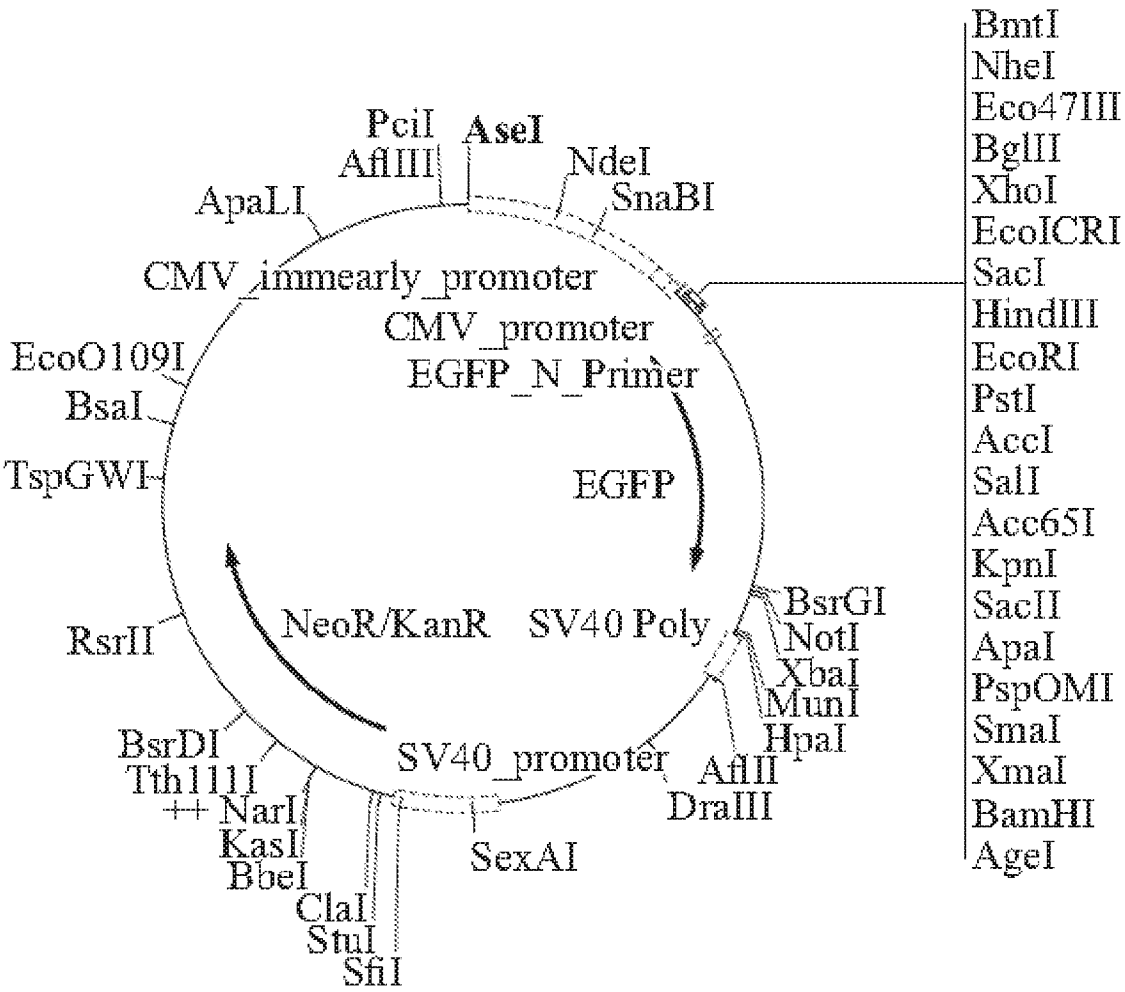

PciI
AflIII
AseI
NdeI
SnaBI
ApaLI
CMV_immearly_promoter
CMV_promoter
EGFP_N_Primer
EcoO109I
BsaI
TspGWI
EGFP
NeoR/KanR
SV40 Poly
RsrII
BsrGI
NotI
XbaI
MunI
HpaI
BsrDI
Tth111I
++ NarI
KasI
BbeI
ClaI
StuI
SfiI
SV40_promoter
AflII
DraIII
SexAI
BmtI
NheI
Eco47III
BglII
XhoI
EcoICRI
SacI
HindIII
EcoRI
PstI
AccI
SalI
Acc65I
KpnI
SacII
ApaI
PspOMI
SmaI
XmaI
BamHI
AgeI

FIG. 9

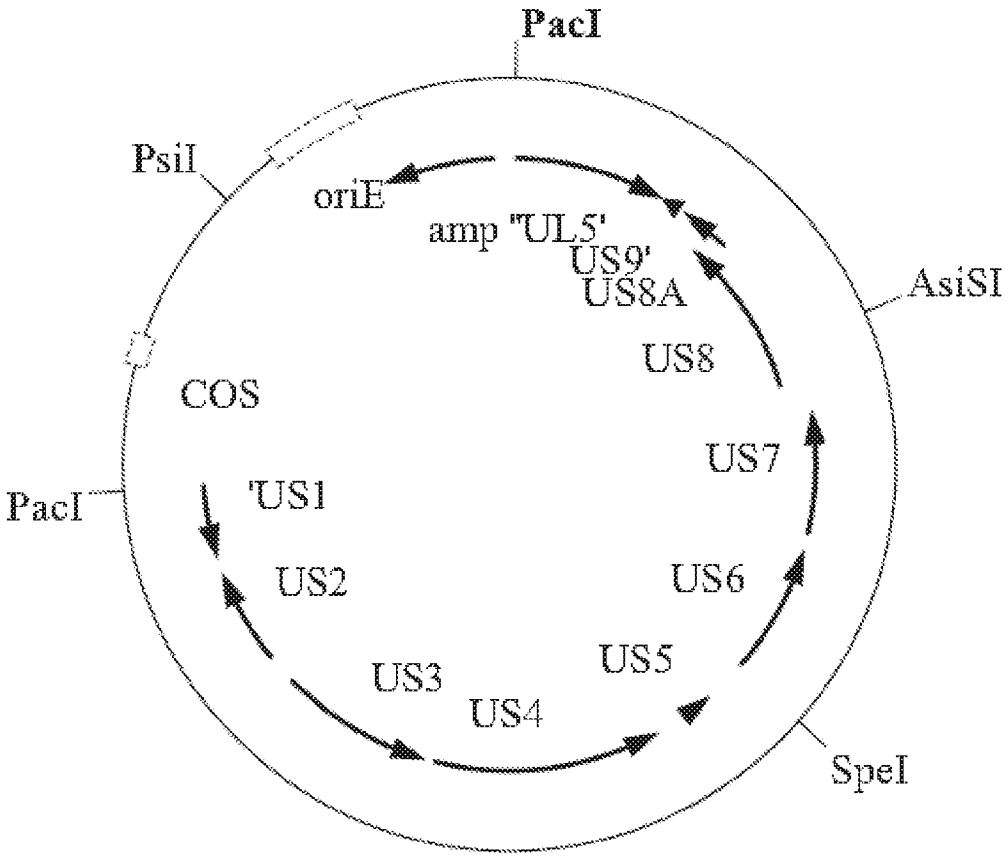

PacI
PsiI
oriE
amp
'UL5'
US9'
US8A
US8
AsiSI
COS
US7
PacI
'US1
US2
US6
US5
US3
US4
SpeI

FIG. 10

FIG. 11A

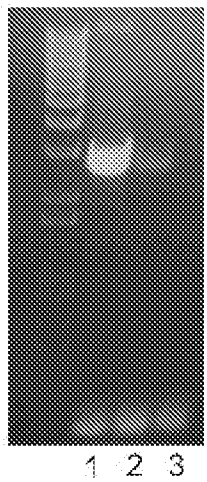


FIG. 11B

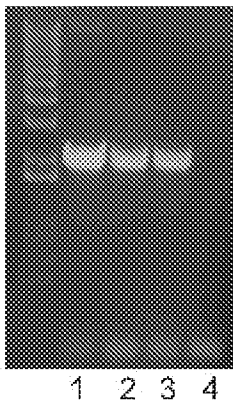
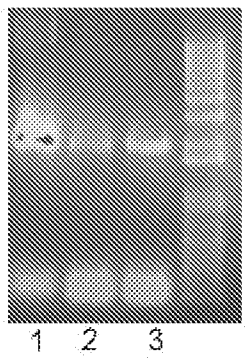


FIG. 11C



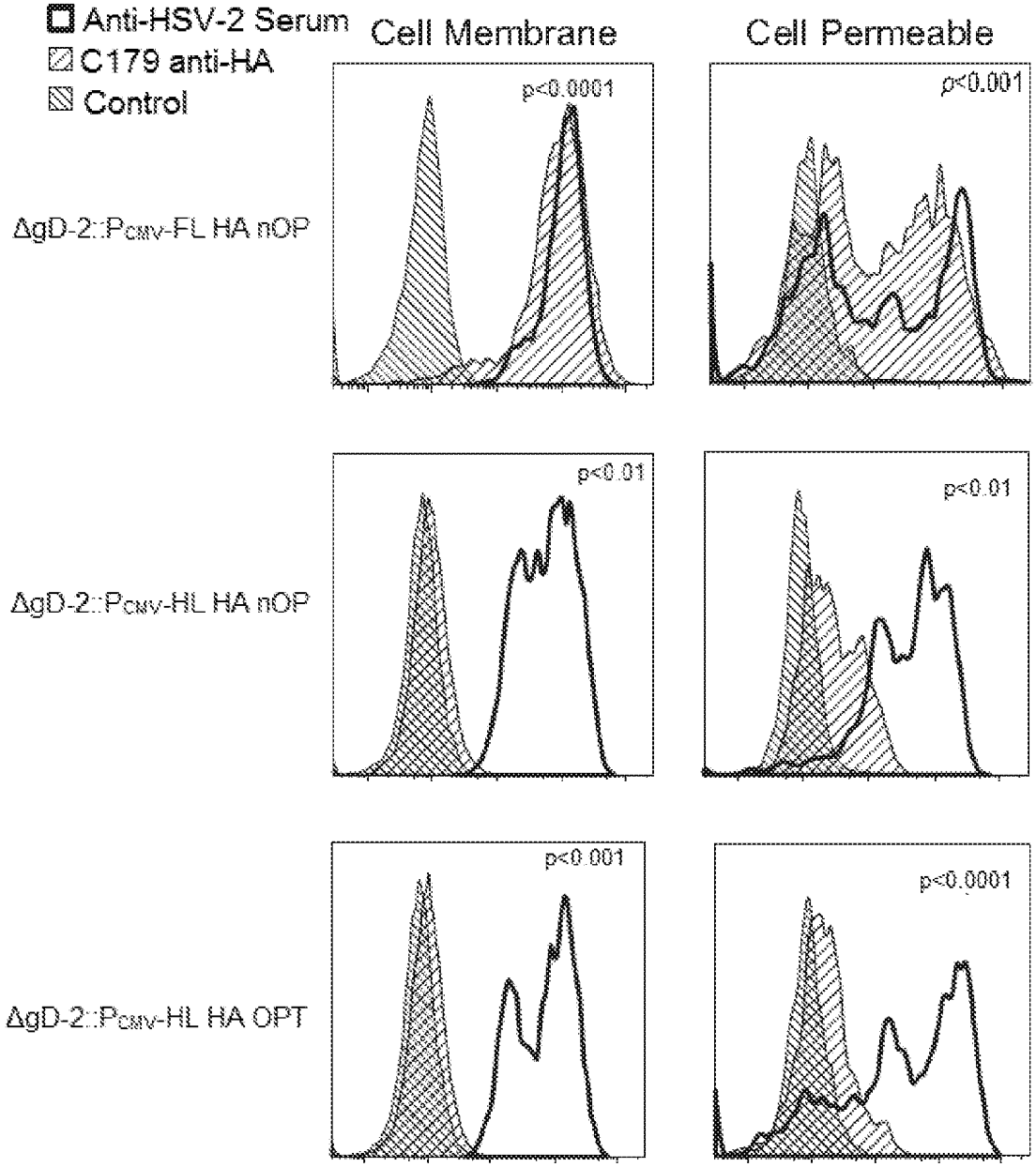

Anti-HSV-2 Serum
C179 anti-HA
Control
Cell Membrane
Cell Permeable
ΔgD-2::PCMV-FL HA nOP
ΔgD-2::PCMV-HL HA nOP
ΔgD-2::PCMV-HL HA OPT
p<0.0001
p<0.001
p<0.01
p<0.01
p<0.001
p<0.0001

FIG. 12

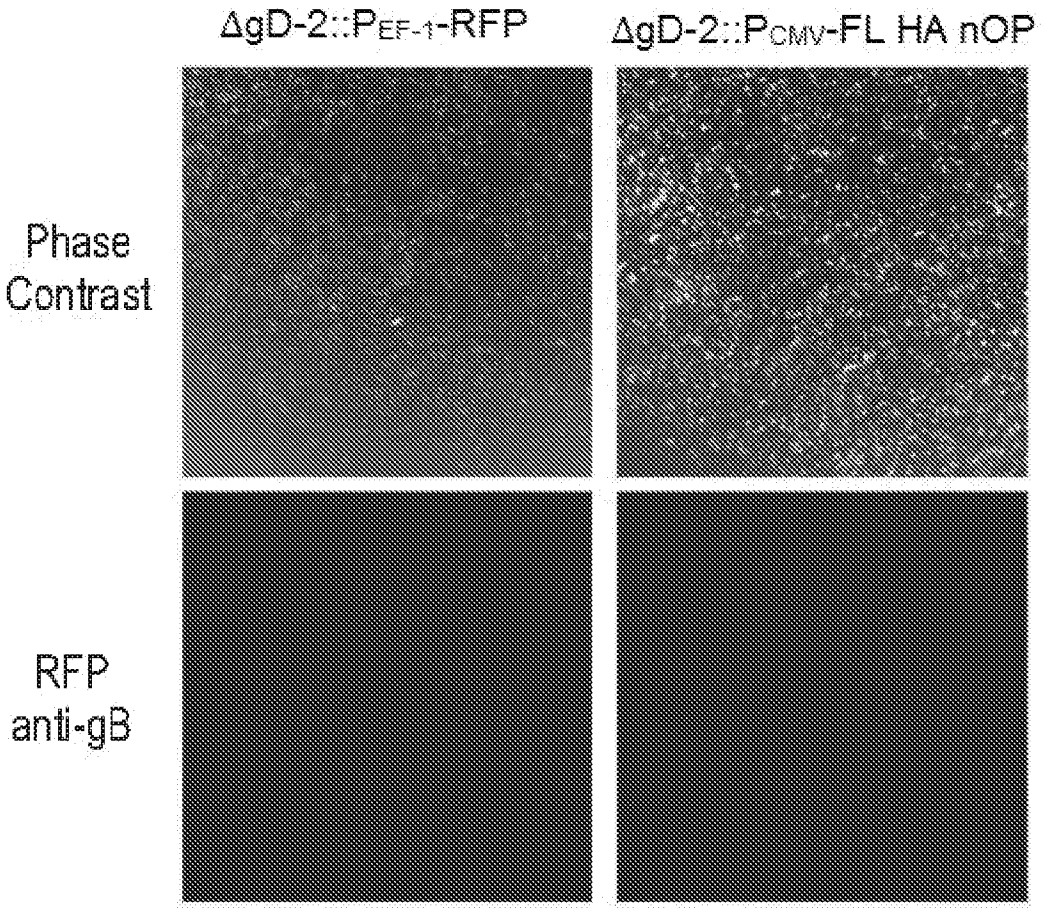


FIG. 13

FIG. 14A

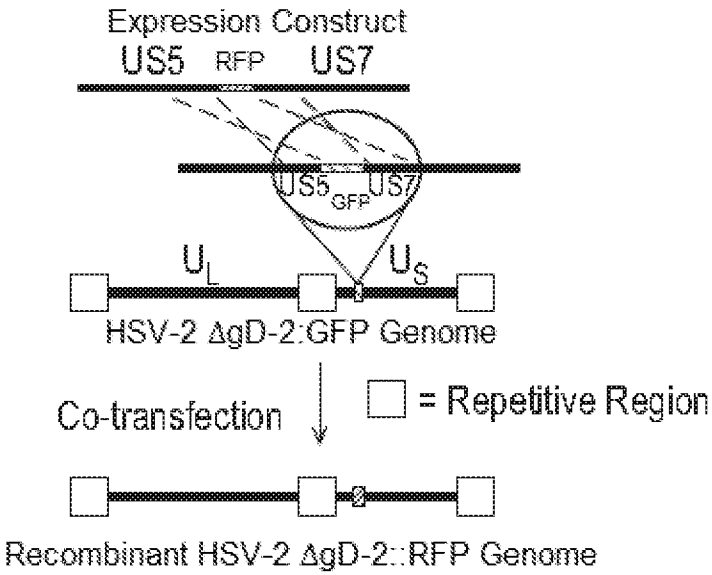


FIG. 14B

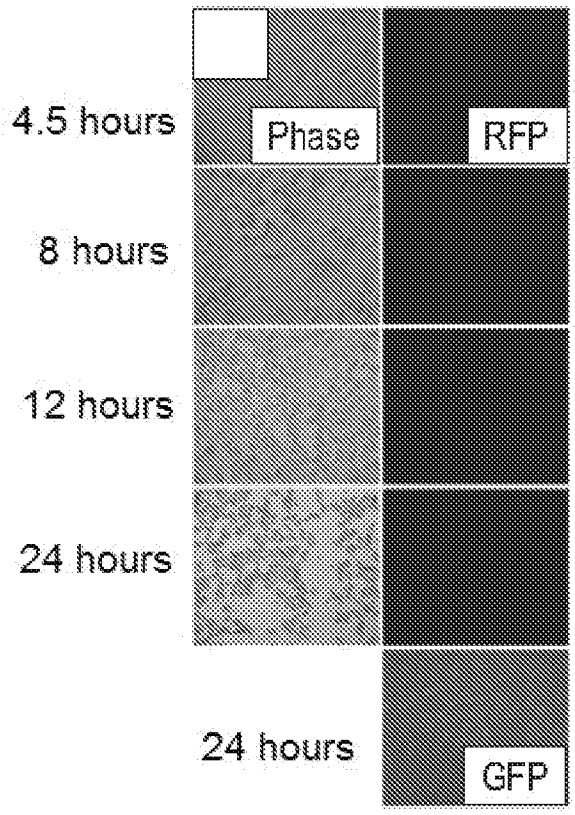


FIG. 14C

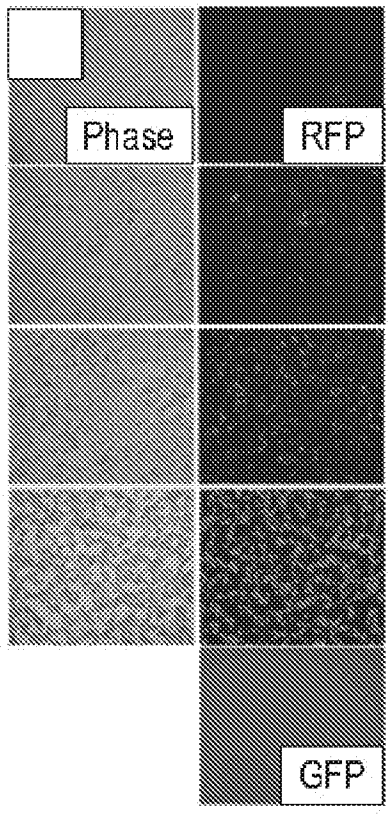


FIG. 14D

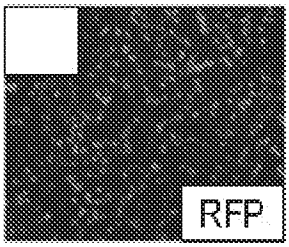


FIG. 14E

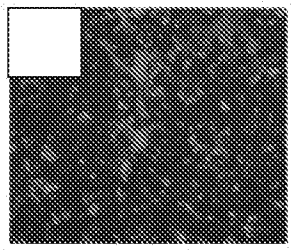


FIG. 15A

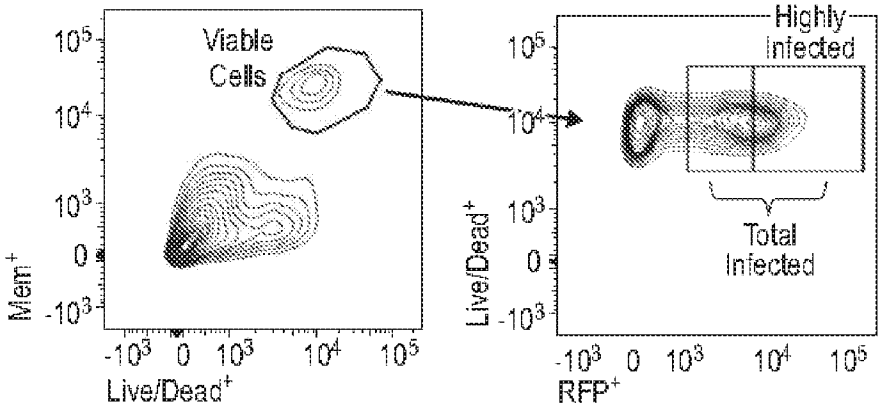


FIG. 15B

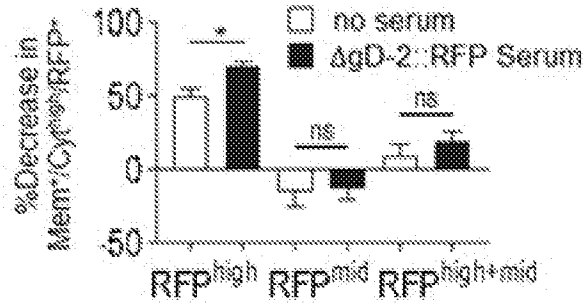
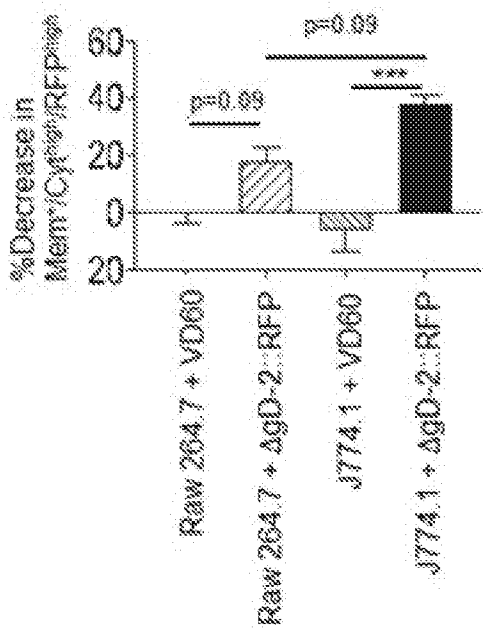


FIG. 15C



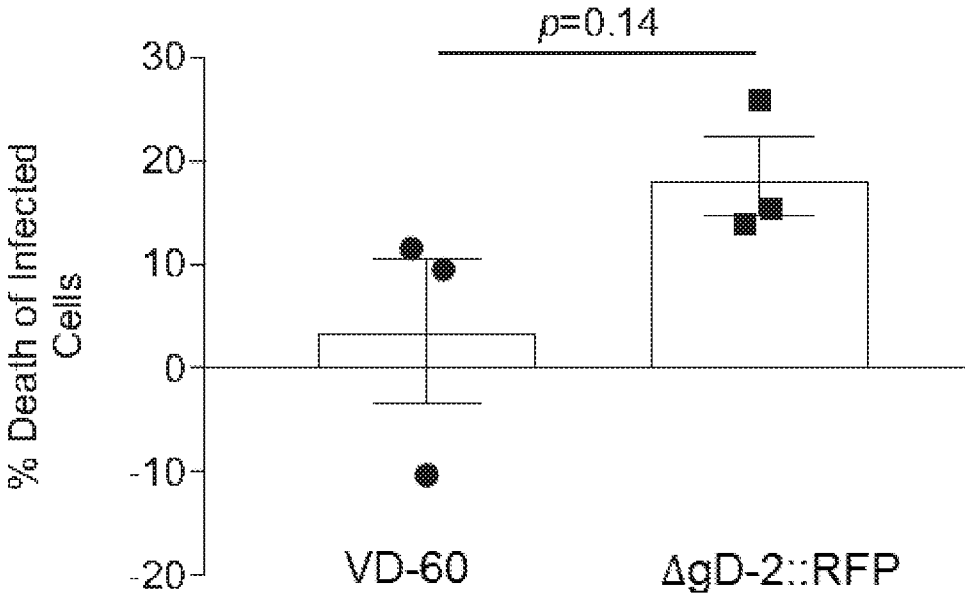


FIG. 16

FIG. 17A

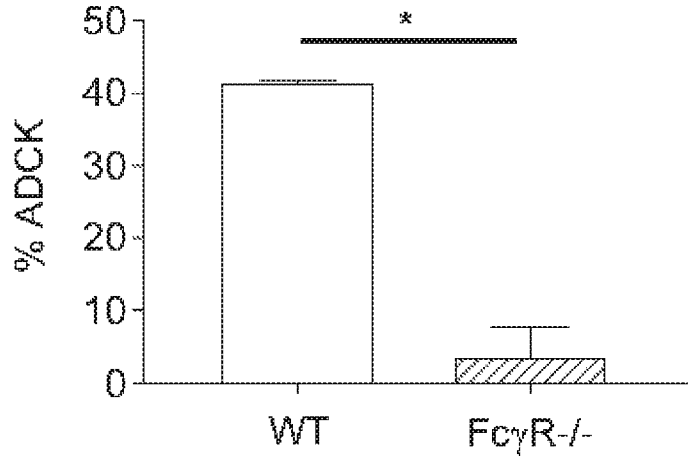
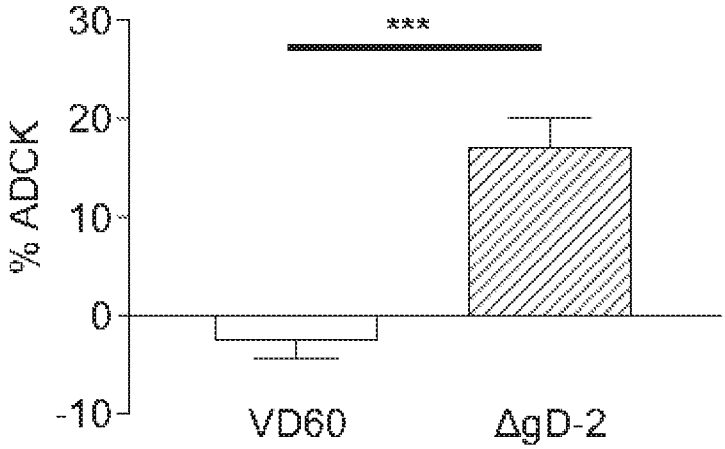


FIG. 17B



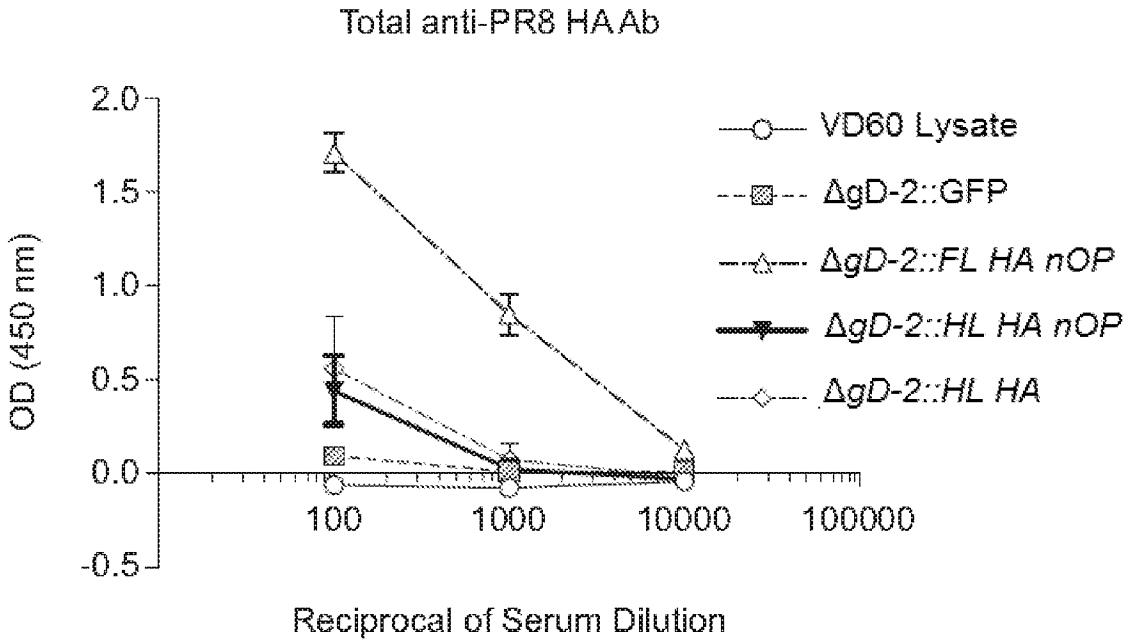

Total anti-PR8 HA Ab
2.0
1.5
1.0
0.5
0.0
-0.5
OD (450 nm)
100
1000
10000
100000
Reciprocal of Serum Dilution
VD60 Lysate
ΔgD-2::GFP
ΔgD-2::FL HA nOP
ΔgD-2::HL HA nOP
ΔgD-2::HL HA

FIG. 18

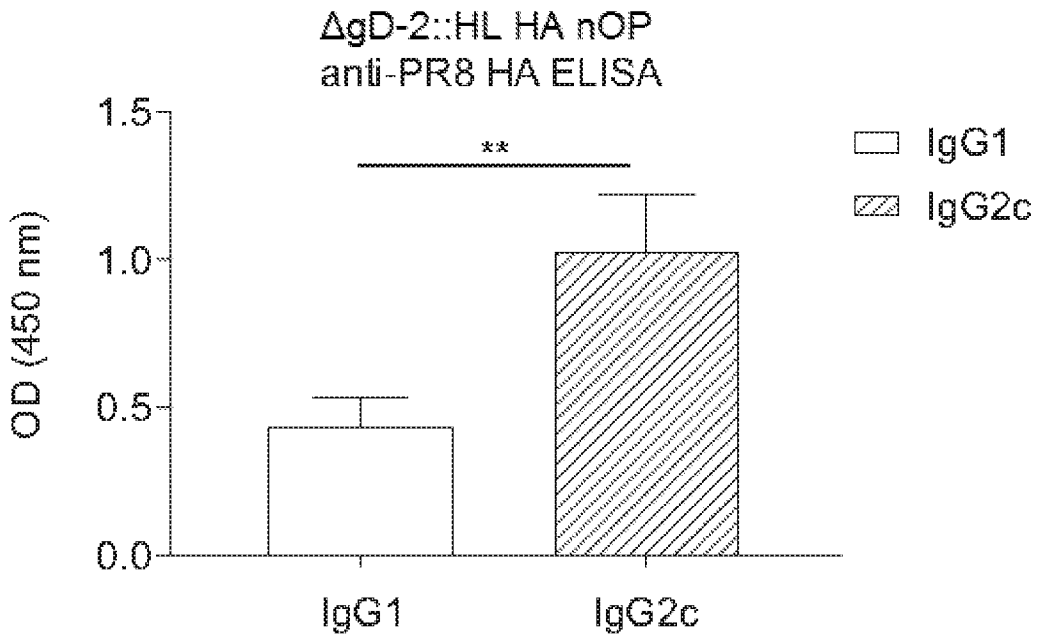

ΔgD-2::HL HA nOP
anti-PR8 HA ELISA
OD (450 nm)
1.5
1.0
0.5
0.0
**
IgG1
IgG2c
IgG1
IgG2c

FIG. 19

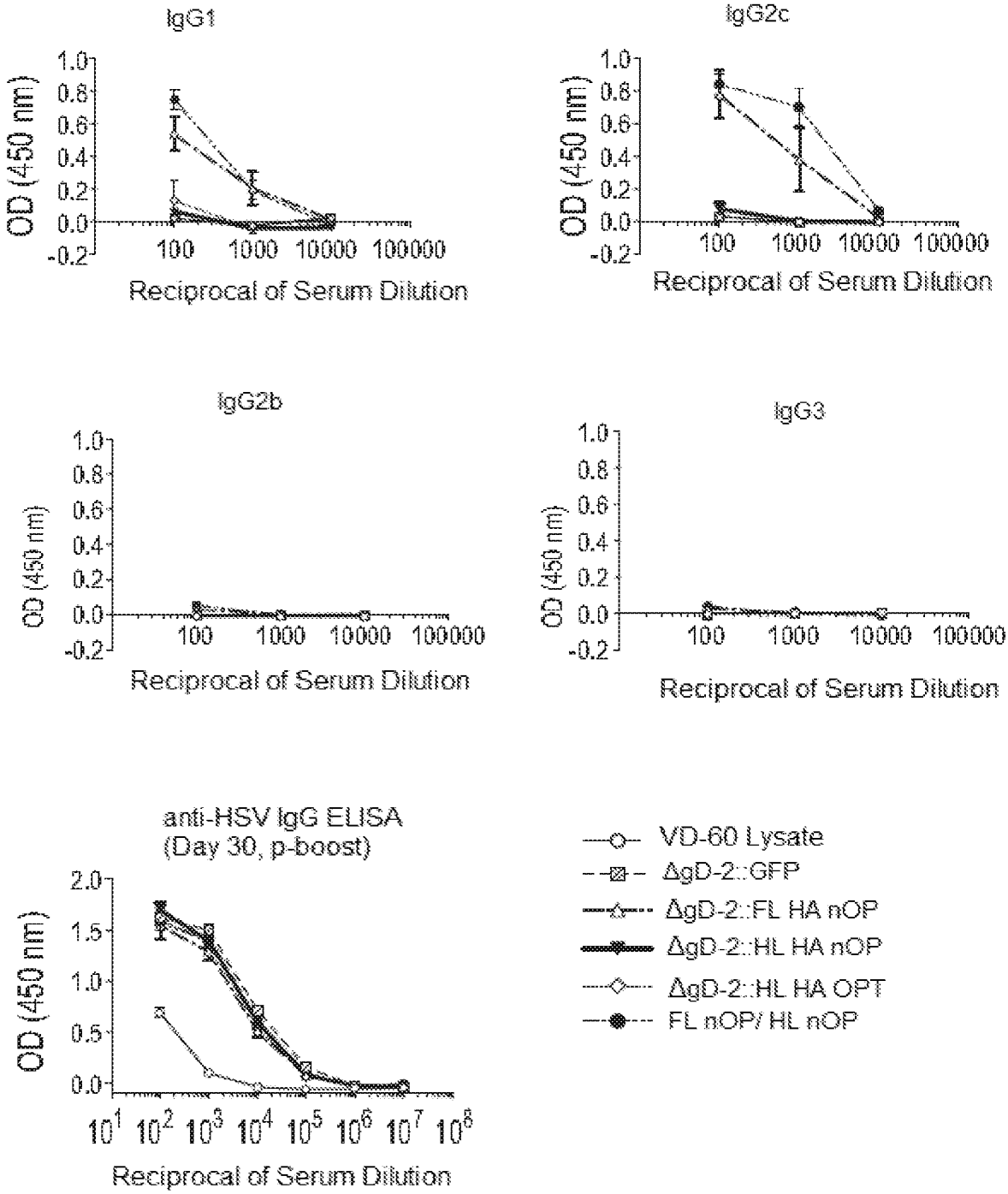

IgG1
OD (450 nm)
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
100
1000
10000
100000
Reciprocal of Serum Dilution
IgG2c
OD (450 nm)
Reciprocal of Serum Dilution
IgG2b
OD (450 nm)
Reciprocal of Serum Dilution
IgG3
OD (450 nm)
Reciprocal of Serum Dilution
anti-HSV IgG ELISA
(Day 30, p-boost)
OD (450 nm)
2.0
1.5
1.0
0.5
0.0
10^1 10^2 10^3 10^4 10^5 10^6 10^7 10^8
Reciprocal of Serum Dilution
VD-60 Lysate
ΔgD-2::GFP
ΔgD-2::FL HA nOP
ΔgD-2::HL HA nOP
ΔgD-2::HL HA OPT
FL nOP/ HL nOP

FIG. 20

FIG. 21A

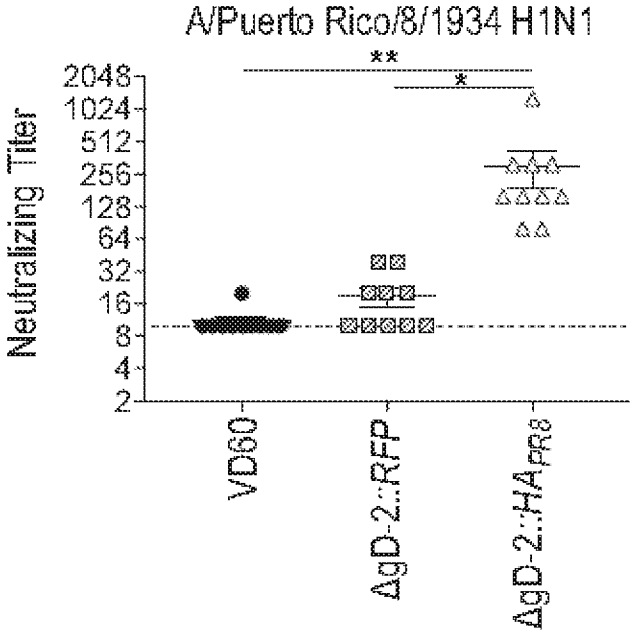


FIG. 21B

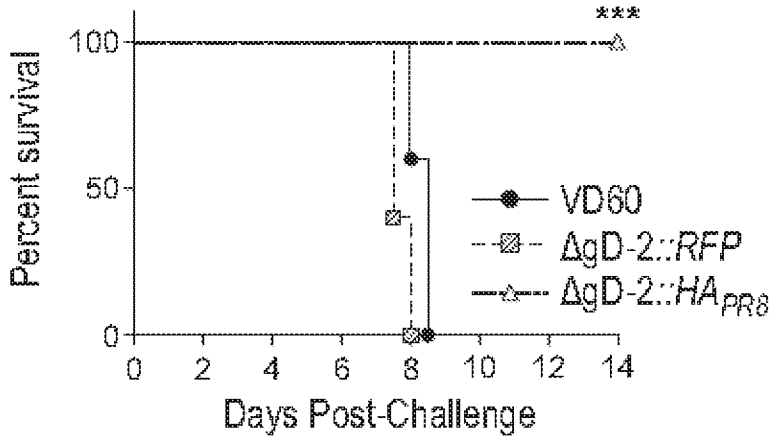


FIG. 21C

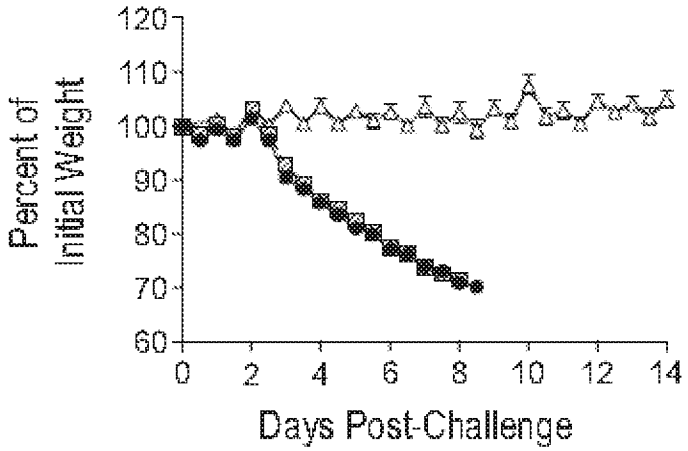


FIG. 22A

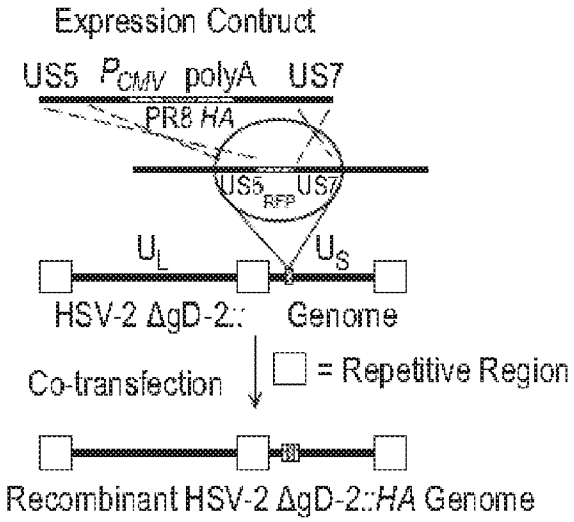
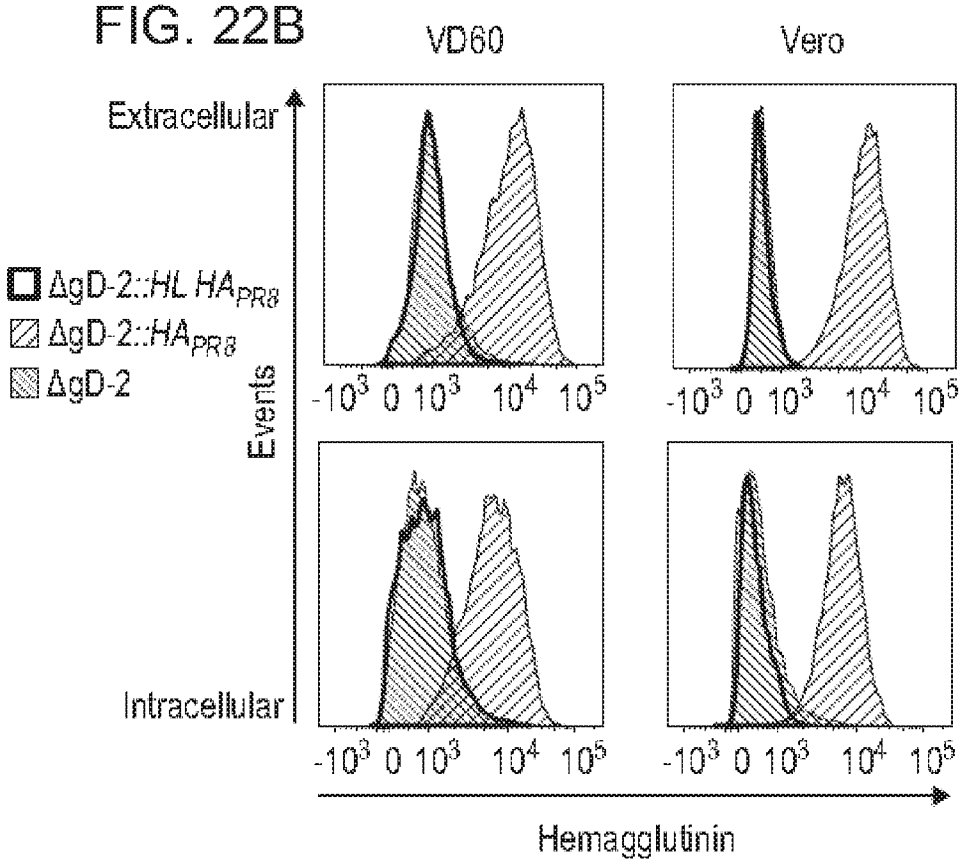


FIG. 22B



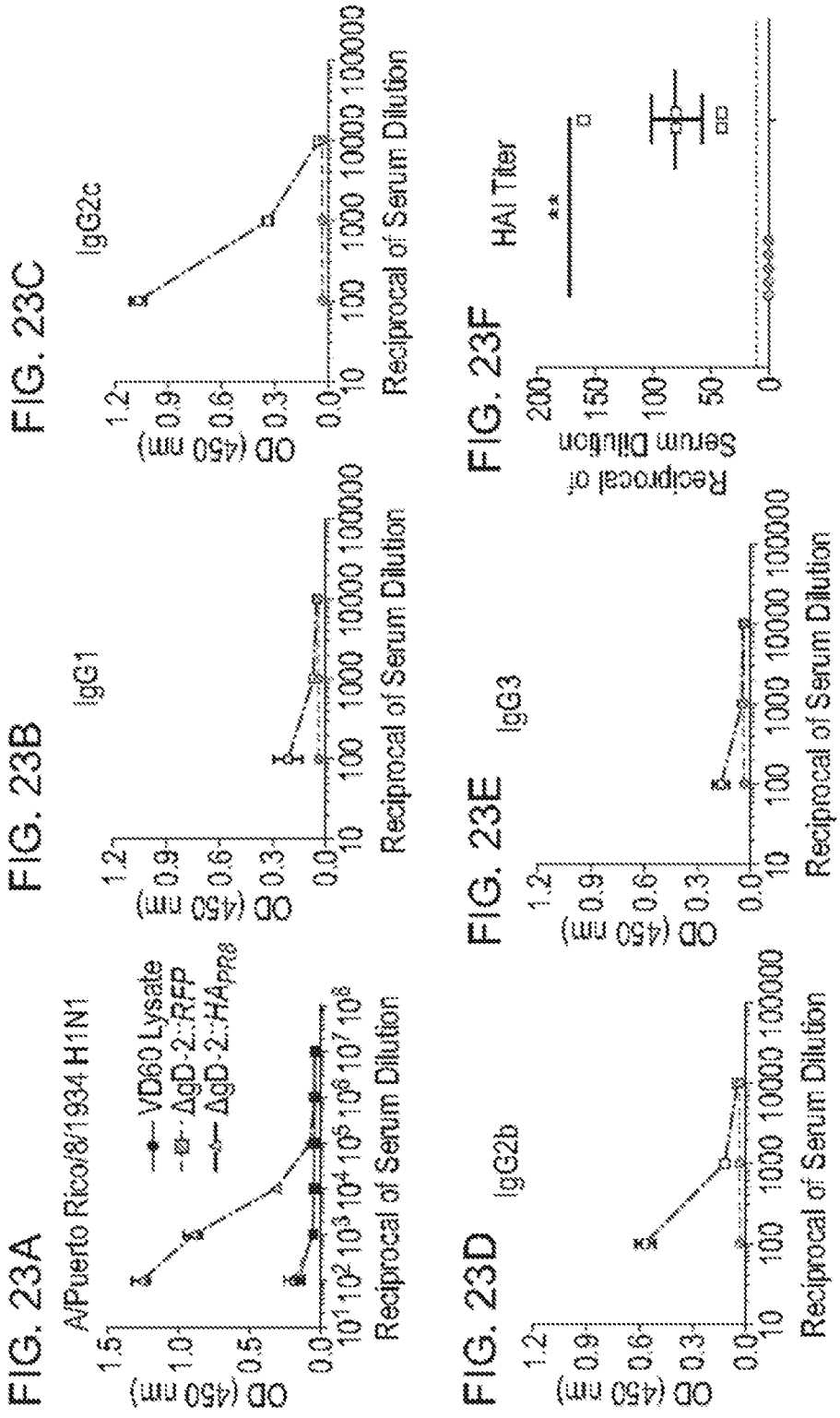


FIG. 23A
A/Puerto Rico/8/1934 H1N1
VD60 Lysate
ΔgD-2::RFP
ΔgD-2::HA$_{PR8}$
OD (450 nm)
Reciprocal of Serum Dilution
FIG. 23B
IgG1
OD (450 nm)
Reciprocal of Serum Dilution
FIG. 23C
IgG2c
OD (450 nm)
Reciprocal of Serum Dilution
FIG. 23D
IgG2b
OD (450 nm)
Reciprocal of Serum Dilution
FIG. 23E
IgG3
OD (450 nm)
Reciprocal of Serum Dilution
FIG. 23F
HAI Titer
Reciprocal of Serum Dilution

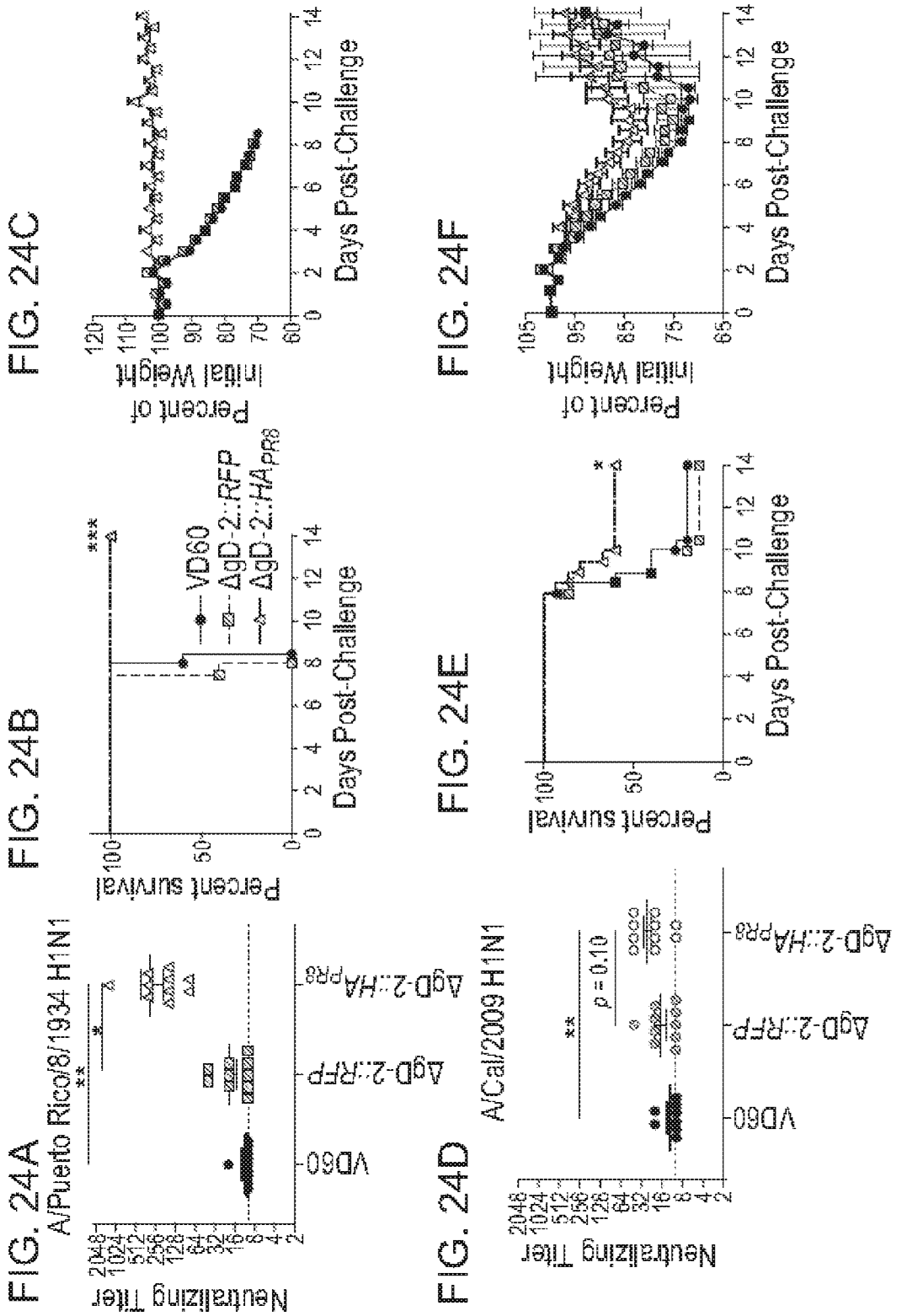


FIG. 24A
A/Puerto Rico/8/1934 H1N1
Neutralizing Titer
VD60
ΔgD-2::RFP
ΔgD-2::HA$_{PR8}$
FIG. 24B
Percent survival
Days Post-Challenge
VD60
ΔgD-2::RFP
ΔgD-2::HA$_{PR8}$
FIG. 24C
Percent of Initial Weight
Days Post-Challenge
FIG. 24D
A/Cal/2009 H1N1
Neutralizing Titer
p = 0.10
VD60
ΔgD-2::RFP
ΔgD-2::HA$_{PR8}$
FIG. 24E
Percent survival
Days Post-Challenge
FIG. 24F
Percent of Initial Weight
Days Post-Challenge

FIG. 25A

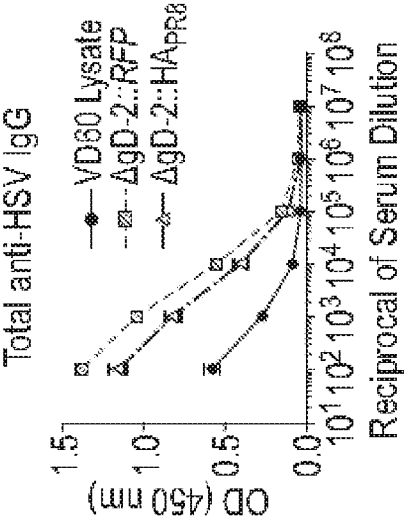


FIG. 25B

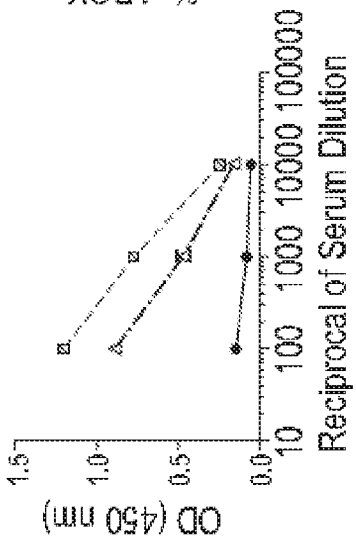


FIG. 25C

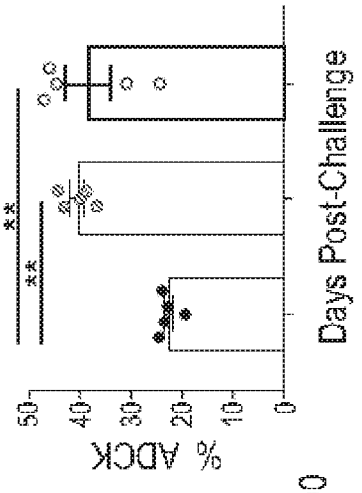


FIG. 25D

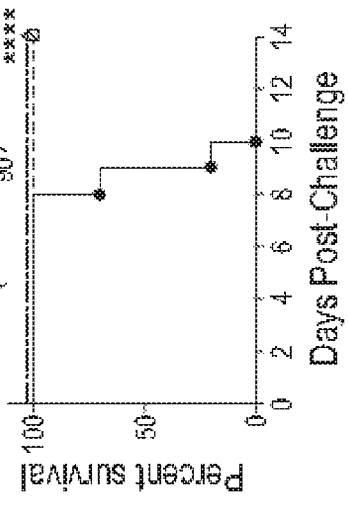


FIG. 25E

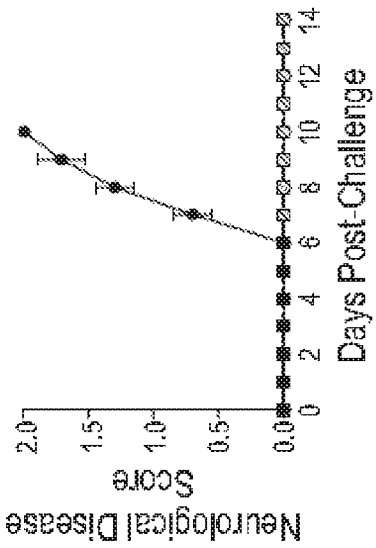
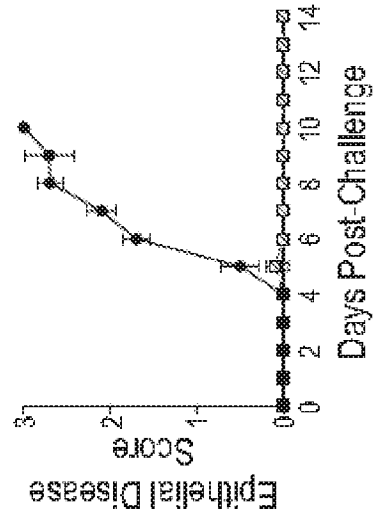


FIG. 25F



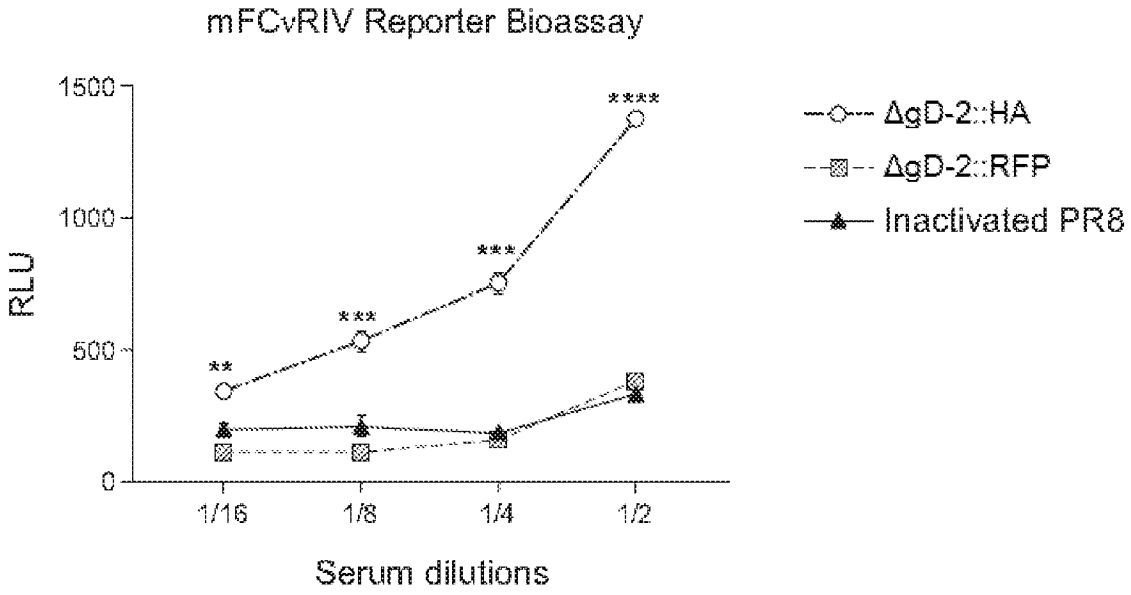

mFCvRIV Reporter Bioassay
RLU
1500
1000
500
0
1/16
1/8
1/4
1/2
Serum dilutions
**
***
***
****
ΔgD-2::HA
ΔgD-2::RFP
Inactivated PR8

FIG. 26

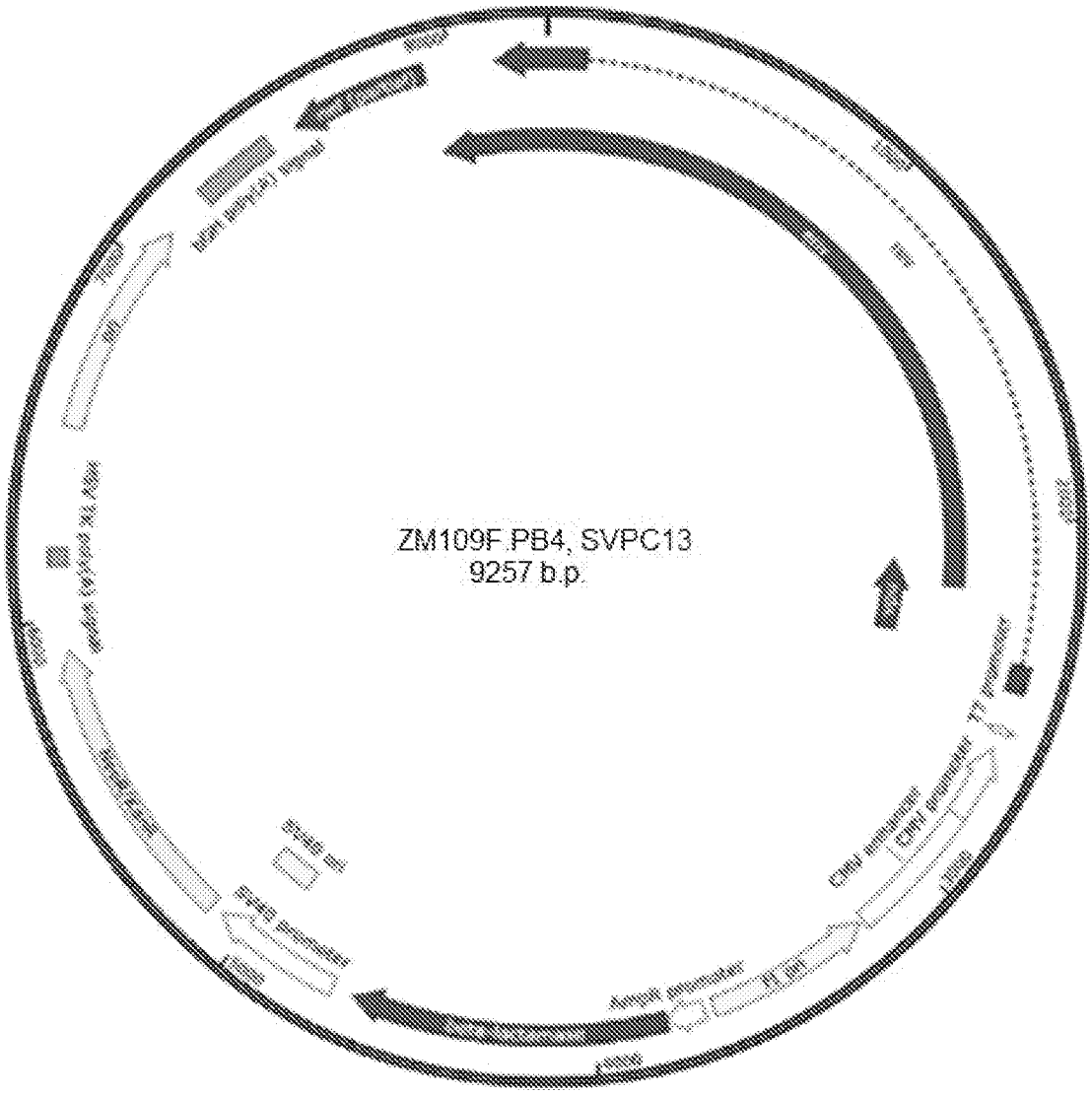


FIG. 27

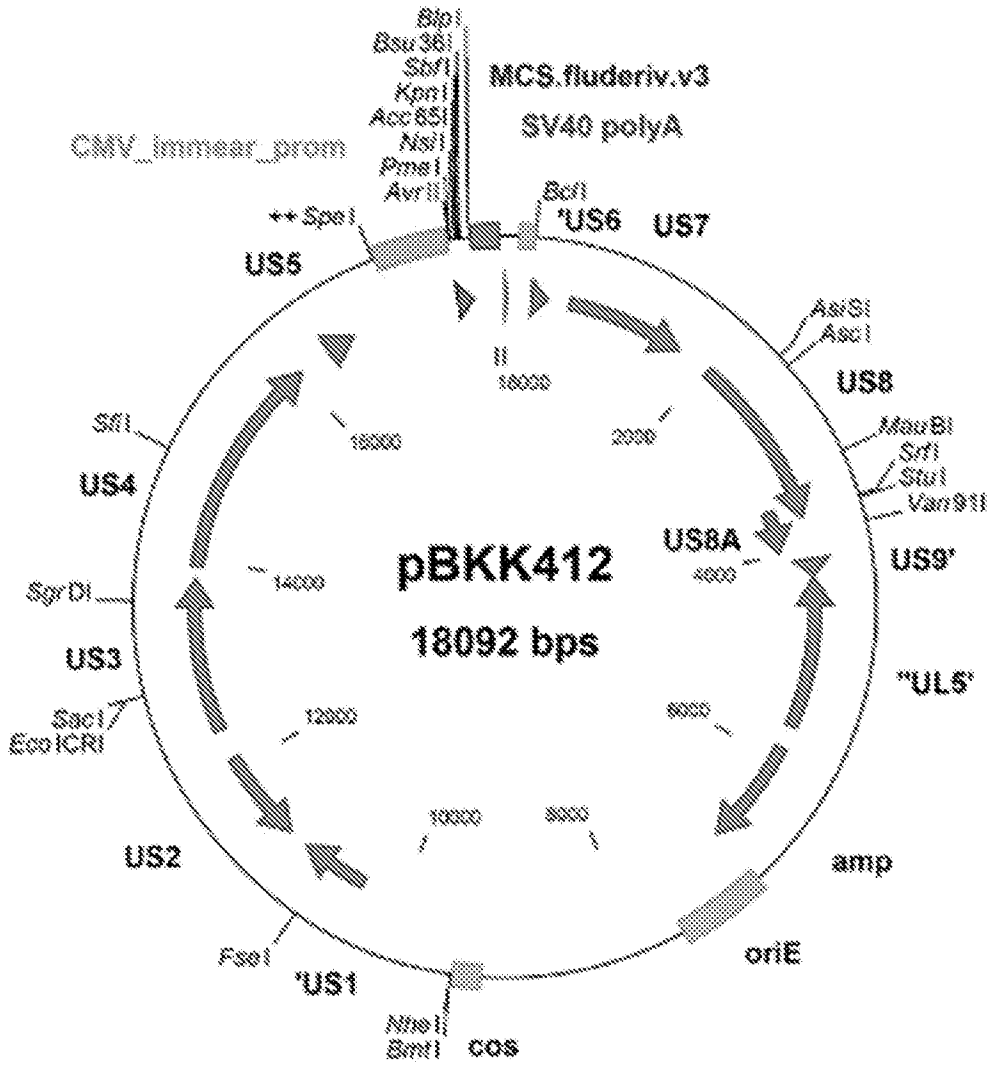

Blp I
Bsu 36I
Sbf I
Kpn I
Acc 65I
Nsi I
Pme I
Avr II
MCS.fluderiv.v3
SV40 polyA
CMV_immear_prom
Bcl I
'US6
US7
++ Spe I
US5
AsiSI
Asc I
US8
II
18000
2000
MauBI
Srf I
Stu I
Van 91I
US9'
US8A
4000
pBKK412
18092 bps
14000
16000
Sfi I
US4
SgrDI
US3
Sac I
Eco ICRI
12000
6000
"UL5'
10000
8000
US2
amp
oriE
Fse I
'US1
Nhe I
Bmt I
cos

FIG. 28

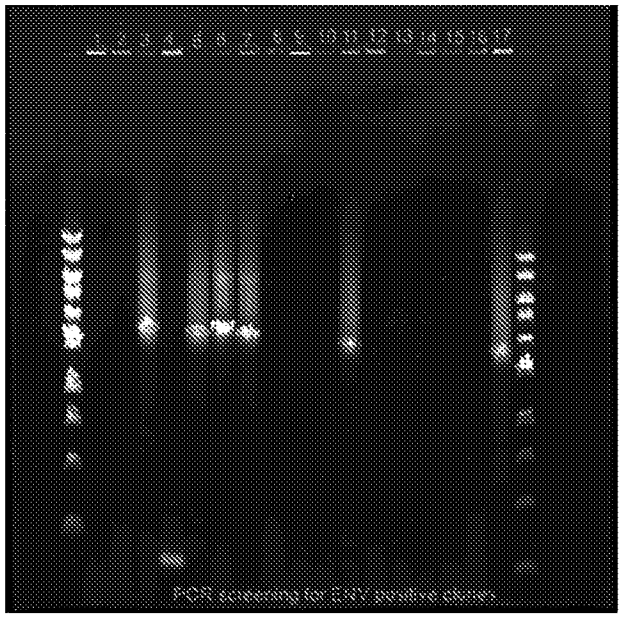


FIG. 29A

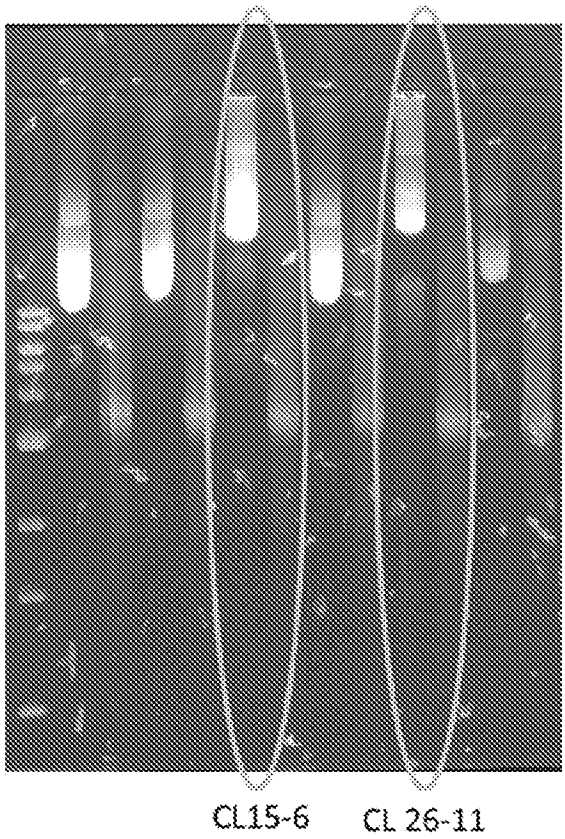


FIG. 29B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 19/30259

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 39/245, A61K 39/12, C12N 7/00 (2019.01)
CPC - C07K 14/005, A61K 2039/525, A61K 2039/5254, A61K 2039/5256, A61K 2039/54, A61K 2039/572, C12N 2710/16621, C12N 2710/16634, C12N 2710/16643, C12N 2710/16662

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---------- Y	US 2017/0202952 A1 (ALBERT EINSTEIN COLLEGE OF MEDICINE, INC) 20 July 2017 (20.07.2017) para [0017]; para [0024]; para [0052]; para [0067]; para [0109]; para [0122]; para [0131]; para [0143]; para [0161]	49-51 ---------- 1-3, 16-18
Y	US 2015/0232812 A1 (BIOVEX LIMITED) 20 August 2015 (20.08.2015) para [0037]; para [0039]; para [0041-0042]	1-3, 16, 18/16
Y	US 2013/0028924 A1 (ERTL et al.,) 31 January 2013 (31.01.2013) Abstract; Claim 1; Claim 16; para [0041]	17, 18/17

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 June 2019	17 JUL 2019

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 19/30259

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4-15, 19-48, 52-58
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.