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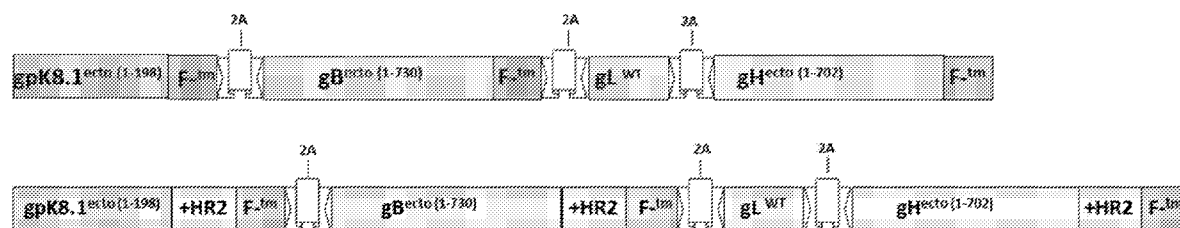
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(54) Title: MULTIVALENT KAPOSI SARCOMA-ASSOCIATED HERPESVIRUS-LIKE PARTICLES AND USES THEREOF

Fig. 1A



(57) Abstract: Disclosed are vaccine compositions comprising a single KSHV-LP comprising two or more KSHV glycoproteins and/or one or more T cell antigens and methods of preventing or treating KSHV infections using the vaccine compositions. Also disclosed is an expression system or a single expression vector for co-expressing two or more KSHV glycoproteins simultaneously to generate a vaccine comprising a single virus-like particle. The expression system may include a single plasmid inserted with two or more nucleic acid sequences that encode two or more KSHV glycoproteins linked by one or more linking sequences such that the KSHV glycoproteins are co-expressed simultaneously.



MULTIVALENT KAPOSI SARCOMA-ASSOCIATED HERPESVIRUS-LIKE PARTICLES AND USES THEREOF

PRIORITY CLAIM

[0001] The present invention claims the benefit of U.S. Provisional Patent Application No. 62/809,481, filed February 22, 2019, the content of which is incorporated herein by reference in its entirety.

GOVERNMENT INTEREST

[0002] This invention was made with government support under Grant No. K01CA184388, awarded by the National Institutes of Health (NIH). The government has certain rights in the invention.

SEQUENCE LISTING

[0003] This application contains a Sequence Listing, which was submitted in ASCII format via EFS-Web, and is hereby incorporated by reference in its entirety. The ASCII copy, created on February 20, 2020, is named SequenceListing.txt and is 11.0 kilobytes in size.

BACKGROUND

[0004] Infection with Kaposi sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus-8 (HHV-8) is estimated to account for over 44,000 new cancer cases and 20,000 deaths globally every year (1). KSHV is associated with the endothelial-based Kaposi sarcoma (KS) and two rare B cell-based lymphomas: primary effusion lymphoma and multicentric Castleman disease, as well as KSHV inflammatory cytokine syndrome (2-5), particularly in immunosuppressed patients. KS is the most common cancer among persons with AIDS and is often incurable with current treatment options. Unlike other human herpesviruses (HHVs), KSHV is not ubiquitous but is highly prevalent in some areas, such as sub-Saharan Africa where KS is the leading cancer among people living with HIV/AIDS (1, 2, 6). Despite the HIV/AIDS pandemic and KSHV endemicity in Africa, the Mediterranean region and some parts of South America, efforts to develop a prophylactic and/or therapeutic vaccine against KSHV and its associated malignancies have been limited, with no

clinical vaccine trial ever reported (7). Hence, there is a need for a vaccine to prevent and treat KSHV infections.

BRIEF DESCRIPTION OF THE DRAWINGS

[0005] Figures 1A-1B show construction and expression of polycistronic plasmids expressing four KSHV glycoproteins. Figure 1A shows schematic representation of pCAGGS-gpK8.1-F-2A-gB-F-2A-WTgL-2A-gH-F-/+HR2 plasmids (not drawn to scale). A single transcript was synthesized to express the gpK8.1 ectodomain (ED) fused to NDV fusion protein (F) transmembrane/cytoplasmic (TM/CT) domains (gpK8.1-F), the gB ED fused to NDV F TM/CT (gB-F), and the gH ED fused to NDV F TM/CT (gH-F), each with or without heptad region 2 (+/-HR2), and with full-length wild-type gL (WTgL) inserted between the gB and gH sequences. Sequence fidelity was confirmed using Sanger sequencing. The numbers shown represent corresponding amino acid numbers of the EDs. Figure 1B shows expression of gpK8.1-gB-WTgL-gH-/+HR2 in HEK-293 and CHO cells. 10⁶ cells from HEK-293 or CHO cells were seeded in a six-well plate and transfected with 2 µg of pCAGGS-gpK8.1-F-2A-gB-F-2A-WTgL-2A-gH-F-/+HR2 or pCAGGS-gpK8.1-F+HR2 (positive control). Transfection efficiency was evaluated 48 hours post-transfection by staining transfected HEK-293 and CHO cells with gpK8.1 mAb, followed by secondary antibody goat-anti-mouse IgG conjugated to AF488. Unstained cells, cells stained with secondary antibody alone (isotype control), and untransfected cells served as negative controls, and gpK8.1-transfected cells served as positive control. Cells were analyzed using FACS by acquiring 10,000 events.

[0006] Figures 2A-2D show generation of stable CHO cells expressing gpK8.1-gB-WTgL-gH-/+HR2 proteins and production and characterization of purified KSHV-LPs. Figure 2A shows generation of stable CHO gpK8.1-gB-WTgL-gH-/+HR2 cells. CHO cells were transfected with 2 µg of pCAGGS-gpK8.1-2A-gB-2A-WTgL-2A-gH-F-/+HR2. Forty-eight hours post-transfection, cells were maintained in selection media supplemented with puromycin (5 µg/ml) until colonies of puromycin-resistant cells were formed. Stable cells were harvested, re-suspended in 1 x PBS and stained as in Figure 1B. Stained cells were live-sorted using FACS five times to a purity of ~50% of K8.1-expressing cells. Figure 2B is a schematic showing of the production of KSHV-LPs. KSHV gpK8.1-gB-WTgL-gH-/+HR2 stable CHO cells were co-

transfected with pCAGGS NDV NP and pCAGGS NDV M plasmids. Supernatants from transfected cells were collected and clarified and KSHV-LPs were pelleted and sucrose-gradient purified as described in the working examples. Figure 2C shows the immunoblot analysis of purified KSHV-LPs. Un-transfected CHO cells, KSHV gpK8.1-gB-WTgH-gL-gH⁺/-HR2 stable CHO cells (CHO KSHV-LPs⁺/-HR2), and purified KSHV-LPs⁺/-HR2 were resolved on a 4–12% SDS polyacrylamide gel, transferred to a polyvinylidene fluoride membrane, and analyzed by immunoblot using rabbit polyclonal (anti-2A or anti-NDV) or mouse monoclonal (anti-gpK8.1 or anti-gH/gL) antibodies as indicated. Figure 2D shows the structural and morphological characterization of KSHV-LPs by TEM. rKSHV-eGFP.219 and KSHV-LPs⁺/-HR2 were fixed in 4% paraformaldehyde and absorbed to glow-discharged, carbon-coated, 200-mesh EM grids. EM images were collected using an FEI Tecnai 12 TEM and recorded with a Gatan 2×2 k CCD camera at a magnification of 21,000X and a defocus value of ~1.5 μm .

[0007] Figures 3A-3C show KSHV-glycoprotein-specific IgG titers in immunized New Zealand white rabbits. Figure 3A shows Coomassie stain (left) and immunoblot (right) of KSHV gpK8.1, gB, and gH/gL recombinant Fc-His tagged proteins. Fc-6xHis-tagged recombinant KSHV gpK8.1, gB, and gH/gL proteins were expressed by transiently transfecting HEK-293 6E cells. Culture media were harvested six days post-transfection by centrifugation and filtration through a 22- μm filter. The Fc-6xHis-tagged KSHV proteins in the media were purified using Protein A spin columns, concentrated in PBS using Amicon Ultra 15 centrifugal filter units, and quantified using a nanodrop spectrophotometer. To confirm the specificity of the proteins, the concentrated proteins were separated on a 4–12% SDS-PAGE and detected by Coomassie blue stain (for molecular weight) or transferred to a polyvinylidene fluoride membrane for immunoblot analysis using monoclonal anti-gpK8.1 or anti-gH/gL or polyclonal goat anti-human Fc (gB) antibodies as indicated. Figure 3B shows immunization and bleeding schedules of wild-type New Zealand white rabbits. Eight- to 10-week-old rabbits (n=6/treatment) were immunized subcutaneously at Days 0, 28 and 42 with 50 μg of purified KSHV-LPs⁺/-HR2, UV-inactivated KSHV, or TNE buffer, all adsorbed to alum and MPL as adjuvants. Immunized rabbits were bled seven days pre-immunization (-7) and on Days 14, 35, 49, 70, and 90 (terminal bleed). Figure 3C shows serum KSHV glycoprotein-specific antibody responses. KSHV-glycoprotein

IgG-specific antibody titers in sera from immunized rabbits were determined using ELISA with recombinant tagged gpK8.1, gB, and gH/gL proteins; results of quadruplicate replicates for each of the six animals per group are expressed as mean $\pm$ standard deviation (SD). Differences in antibody titers between all groups were analyzed using a Kruskal-Wallis test; differences between the +HR2 and +HR2 vaccine were assessed using a Mann-Whitney test.

[0008] Figures 4A-4B show *in vitro* neutralization capabilities of purified IgGs from rabbits immunized with KSHV-LPs+/-HR2 in diverse cell types. Figure 4A shows titration of purified IgGs specific to KSHV glycoproteins by ELISA. Purification of IgG antibodies was conducted by pooling equal amounts of Day 49 sera from immunized rabbits (n=6) from each of the four treatment/control groups. KSHV-glycoprotein IgG-specific antibody titer was determined using ELISA with recombinant tagged proteins gpK8.1, gB, and gH/gL as targets; results of quadruplicate replicates are expressed as mean $\pm$ SD. Figure 4B shows rKSHV-eGFP.219 titration and neutralization assays in epithelial, fibroblast, endothelial, and B cells. Panel i. rKSHV-eGFP.219 virus titration in diverse cell types. Individual cell lines were seeded overnight at a density of 5×10^5 in quadruplicate in 48-well-plates. The individual cell lines were then incubated with 5, 10, 20, 30, or 50 μ l of the purified virus in a total volume of 100 μ l of virus plus serum-free media for 24 hours at 37°C. Infected cells (eGFP+) were quantified using FACS by acquiring a total of 10,000 events. Panel ii. Neutralization activity in HEK-293 cells, HFF-1 cells, HUVEC, and MC116 B cell lines. Neutralization activities were determined by incubating rKSHV-eGFP.219 virus and purified IgGs of varying concentrations (12.5, 25, 50, or 100 μ g/ml) from all groups of test animals for 1 hour at 37°C, before being added to previously seeded cells in a 48-well plate. After 1-hour incubation at 37°C, the cells were thoroughly washed three times with 1 $\times$ PBS before adding growth media. The level of neutralization was determined in the cells after 24 hours using FACS to quantify the number of eGFP+ cells. Cells incubated with virus or with media alone served as positive and negative controls, respectively. Results of quadruplicate replicates are expressed as mean $\pm$ SD.

[0009] Figure 5 shows the construction of various MVA vector constructs expressing multiple KSHV antigens. Figure 5 shows schematic diagram of MVA-KSHV-5Ag-VLP construct, it can be obtained by a 2A-linked polycistronic expressing transcript including the gpK8.1 ectodomain (ED) fused to NDV fusion protein (F)

transmembrane/cytoplasmic (TM/CT) domains (gpK8.1-F); the gB ED fused to NDV F TM/CT (gB-F); and the gH ED fused to NDV F TM/CT (gH-F) without heptad region 2 (-HR2); and full-length wild-type gL (WTgL) between gB and gH, can be inserted at G1L/18R intergenic region of MVABAC-TK. Additionally, the IGR3 site inserted with NDV NP (28 amino acids) fused with partial LANA1 (937-1124 amino acids) protein of KSHV and to facilitate VLPs formation the Del3 site inserted with NDV M. Promotor mH5 can be utilized for expression of all the transgene in MVABAC-TK.

DETAILED DESCRIPTION

[0010] Expression systems, vectors, vaccines for use in preventing or treating Kaposi sarcoma-associated herpesvirus (KSHV) infections and KSHV-associated conditions or malignancies are provided herein. The polyvalent single KSHV-LP vaccine, which is described in detail below, can stimulate the production of neutralizing antibodies and generate both prophylactic and therapeutic antiviral responses against KSHV infections and KSHV-associated malignancies.

[0011] In one aspect, disclosed herein is a novel subunit vaccine that incorporates KSHV envelope glycoproteins required for viral entry in diverse cell types such as gpK8.1, gB, and gH/gL into a single multivalent KSHV-like particle (KSHV-LP). The purified KSHV-LPs were similar in size, shape, and morphology to KSHV virions. In a related aspect, disclosed herein is a method of preventing or treating a subject who is at an elevated risk of KSHV infections and KSHV-associated malignancies or who suffers from KSHV infections and KSHV-associated malignancies. The method comprises administering a therapeutically effective amount of the vaccine disclosed herein to the subject. In yet another related aspect, disclosed herein is a method of neutralizing KSHV infection in vitro in epithelial cells, endothelial cells, fibroblast cells, and B cells, comprising contacting the cells with the KSHV-LPs disclosed herein such that neutralizing antibodies are produced in the cells. In some embodiments, the subject is immunocompromised. In some embodiments, the subject suffers from one or more cancer. In some embodiments, the vaccine further comprises one or more additional glycoproteins such as gM, gN, and ORF4, and/or T cell antigens such as LANA1. The additional glycoproteins and/or T cell antigens can be co-expressed from the same plasmid expressing the KSHV envelope glycoproteins including gpK8.1, gB, and gH/gL or expressed by one or more different

plasmids. In some embodiments, the additional glycoproteins and/or T cell antigens can be incorporated into the same, single multivalent KSHV-LP comprising gpK8.1, gB, and gH/gL. In some embodiments, the vaccine is administered to the subject with one or more adjuvants. In some embodiments, one or more booster doses of the vaccine is administered. One of ordinary skill in the art can optimize the doses, intervals, routes of the vaccine administration to achieve the desired immunogenic effects. Several delivery vectors such as modified vaccinia Ankara vectors and adenovirus vectors can be used to deliver KSHV-LPs as live-attenuated vaccines and to elicit high titers of potent neutralizing antibodies that block infection in both in vitro and in vivo infection models better than those found in UV-KSHV immunized rabbits.

[0012] Modified vaccinia Ankara (MVA) virus is an attenuated vaccine of a poxvirus (Orthopoxvirus family) generated by about 570 serial passages of the vaccinia Ankara strain on chicken embryo fibroblasts (50). MVA was successfully used in eradication of small pox and elicits durable antibody (Ab) responses (51). Administration of a higher dose of MVA (1×10^8 TCID₅₀) was safely tolerated in humans (52, 53). MVA and MVA-vectored vaccines displayed a high safety profile, as they are replication-defective in humans, can only replicate in avian cells and are used for propagation (54). MVA has large genome size (~135kbp) and has lost many of the genes during attenuation, capable of expressing large and multiple transgenes, facilitating expression of KSHV and NDV genes for the formation of KSHV VLPs. Disclosed herein is an MVA-based vaccine design construct to express the KSHV proteins on the surface of non-infectious VLPs. The KSHV VLPs can be formed by the self-assembly and budding of MVA expressed KSHV proteins (K8.1, gB, gL, gH and LANA1) and NDV F, M and NP proteins. KSHV gPs are main target for inducing neutralizing antibody response and its display on the VLP surface could efficiently initiate antibody response similar to KSHV infection. The LANA1 protein functions as T-cell antigen for initiating cell mediating immune response against KSHV.

[0013] As demonstrated in the working examples, vaccination of rabbits with adjuvanted KSHV-LPs generated strong glycoprotein-specific antibody responses, and purified immunoglobulins from KSHV-LP-immunized rabbits neutralized KSHV infection in epithelial, endothelial, fibroblast, and B cell lines, 60–90% at the highest concentration tested. These findings suggest that KSHV-LPs may be an ideal platform for developing a safe and effective prophylactic KSHV vaccine.

[0014] Also demonstrated in the working examples, the co-expression of a single construct of all four glycoproteins interspersed with a 2A self-cleaving peptide for efficient expression and cleavage under a single CMV promoter, and NDV F, NP, and M proteins resulted in the successful self-assembly and release of KSHV-LPs. Purified KSHV-LPs from CHO cells structurally resembled purified KSHV virions in size, shape, and morphology under transmission electron microscope (TEM). In addition, immunoblot assay confirmed that all four KSHV glycoproteins were incorporated into the KSHV-LPs. These data demonstrate that the multivalent VLP expressing four KSHV glycoproteins can be used as a reagent for immunization of animals. The observed effects of pre-existing immunity to KSHV suggest that immunization with the right vaccine candidate may offer protection from KSHV infection and development of KS: patients who become infected with KSHV before contracting HIV (i.e., before immunosuppression) have a significantly reduced incidence of KS compared with those acquiring KSHV after HIV infection (8, 9). Furthermore, KSHV etiology provides a window of opportunity for prevention with a prophylactic vaccine, even in Africa. In Africa, other endemic HHVs achieve widespread transmission and latent infection within the first year of life infecting up to 76% of children. In contrast, few to no children are infected with KSHV by this age or even by five years of age (10-12). In other parts of the world, where KSHV exposure typically occurs during adulthood, transmission is so poor that either repeated contact with the virus, or immunodeficiency, is required to sustain KSHV at >5% population-wide seroprevalence (13, 14). KSHV superinfection in immunocompetent people is also limited, suggesting that some immune protection is conferred among adults (15). Thus, minimal priming of the immune system by a vaccine prior to infection can be achieved to prevent and/or treat KSHV infection and its associated malignancies in developing countries and possibly eradicate KSHV and associated malignancies from developed countries.

[0015] Like other HHVs, KSHV encodes five conserved glycoproteins: gB (open reading frame (ORF) 8), gH (ORF 22), gL (ORF 47), gM (ORF 39), and gN (ORF 53) (16, 17). In addition, KSHV also encodes for a unique glycoprotein, gpK8.1, which is the major immunodominant glycoprotein on the virion and is believed to modulate viral tropism for B cells. In the current model of KSHV infection, the virus is thought to first attach to host cell receptors through the non-conserved gpK8.1, which then signals

gH/gL to activate the gB fusogen. This infection model is supported by the results of experiments that used neutralizing monoclonal antibodies against gpK8.1 or rKSHV deletion mutants lacking gpK8.1, gB or gH/gL to define the role of each KSHV glycoprotein in infecting various cell types. Because these data provide evidence that different KSHV glycoproteins modulate viral tropism for different cell types, targeting a single glycoprotein in a prophylactic vaccine setting might not completely protect against infection of diverse cell types. The immunization protocol disclosed herein includes gpK8.1, gB, and gH/gL complex into a single vaccine to generate a potent vaccine candidate that elicits neutralizing antibodies blocking KSHV infection of diverse permissive cell types of human origin.

[0016] Selection of an appropriate platform is important and unpredictable. Virus-like particles (VLPs) lack the viral genome and typically assemble from viral structural proteins, forming repetitive arrays that resemble a natural virus. As disclosed herein, this platform allows inclusion of multiple selected surface glycoproteins and intracellular T-cell antigens in a polyvalent vaccine.

[0017] Due to the oncogenic potential of KSHV DNA (3, 29), it is not feasible to use live attenuated KSHV or any form of inactivated KSHV to elicit immune responses in clinical settings. To circumvent this shortcoming, disclosed herein are virus-like particles (VLPs) that individually incorporate the key KSHV glycoproteins (gpK8.1, gB, or gH/gL complex) into a VLP. These VLPs are immunogenic in immunized wild-type BALB/c mice; however, their immunogenicity in diverse cell types were unknown.

[0018] Disclosed herein are the construction, assembly and biochemical characterization of candidate multivalent KSHV-like particle (KSHV-LP) vaccines that incorporate all four key glycoproteins into a single VLP. As demonstrated in the working examples, wild-type New Zealand white rabbits immunized with adjuvanted VLPs unexpectedly elicited strong neutralizing antibodies that prevented in vitro KSHV infection of epithelial cell line, endothelial cell line, fibroblast cell line, and B cell line. Thus, this disclosure relates to a novel vaccine approach with a potential clinical application as a prophylactic vaccine against KSHV infection and its associated malignancies.

[0019] Disclosed herein is an immunogenic multi-subunit vaccine comprising a single KSHV-LP that incorporates multiple KSHV glycoproteins including gpK8.1, gB

and gH/gL on the virion envelope surface. As demonstrated in the working examples, New Zealand white rabbits were immunized subcutaneously and boosted with the KSHV-LP adjuvanted with both aluminum hydroxide and monophosphoryl lipid A. The anti-KSHV-specific glycoproteins antibody responses were determined by ELISA. Neutralizing antibody titers in sera of immunized rabbits were assessed in various cell types in vitro. The Rabbits immunized with KSHV-LPs elicited high titers of KSHV-specific antibodies that neutralized virus infection in diverse cultured cell lines.

[0020] It was previously demonstrated that four glycoproteins, gpK8.1, gB, gH and gL, were highly immunogenic and were able to elicit robust neutralizing antibodies individually and in combination in a mice model (30). However, these antigens were not co-expressed or assembled into a single virus-like particle. As described herein, the expression of the glycoproteins as a single transcript separated by the 2A, self-cleavage peptides, under a single promoter and NDV- M and -NP fused to the KSHV latent protein, LANA1 was sufficient for the production of self-assembling and releasing KSHV-LPs. In addition, the structure of the purified KSHV-LPs resembled that of wild type KSHV in terms of the morphology and size of 100 nm spherical particles with spikes observed under TEM. The inner core of VLP consists of NP fused to LANA1 and surrounded by host cell derived lipid bilayer membrane. Innersurface of membrane is layered with M protein and embedded with transmembrane domain of F protein, that are fused with K8.1, gB or gH glycoprotein of KSHV, displaced on surface of VLP. gL non-covalently bound to N-terminal domain to gH also found on VLP surface.

[0021] Although there was no significant difference in the antibodies titers observed between KSHV-LPs+/-HR2 VLPs, KSHV-LPs-HR2 neutralized KSHV infection better, as shown in Figure 6. The neutralization ability of observed in diverse cell types were comparable to that of UV-KSHV that yielded significant antibody titer. In wild type KSHV, high antibody titers did not translate or correlate to the induction of high titers of neutralizing antibodies. The KSHV-LPs-HR2 vaccine although it generated lower antibody titers, the levels of neutralization of KSHV in vitro was comparable to that of wild type KSHV, while KSHV-LPs+HR2 neutralization levels were lower (Figure 6). Similar results have been observed in other VLP based vaccine, where the production of high titers of antibodies does not always correlate to high neutralizing response (Boigard et al., 2017). Although not bound with any theory,

the difference between KSHV-LPs +HR2 and – HR2 neutralization activity might be linked to the presence of the HR2 domain, which is involved in protein trimerization. The trimerization of the antigens could have masked the epitopes that are responsible for eliciting neutralizing antibodies.

[0022] Thus, disclosed herein is a novel prophylactic and therapeutic polyvalent vaccine comprising two or more KSHV envelope glycoproteins and one or more T cell antigens incorporated into a single virus-like particle. In some embodiments, the vaccine comprises two, three, four, five or more glycoproteins including gpK8.1, gH, gL and gB. In some embodiments, the T cell antigens include LANA1 or an immunogenic fragment thereof. In some embodiments, K8.1, gH/gL or gB or an immunogenic fragment thereof may also show T-cell responses. In some embodiments, in addition to the KSHV glycoproteins and T cell antigens, the vaccine further comprises NDV structural proteins including fusion (F), matrix (M), and nucleocapsid (NP) incorporated into the single VLP. In some embodiments, the vaccine further comprises one or more adjuvants such as alum sulphate and monophosphoryl lipid A.

[0023] In some embodiments, disclosed herein is a single vector co-expressing two or more KSHV glycoproteins including gpK8.1, gB, gH, and gL, with each glycoprotein separated from another glycoprotein by 2A sequence. For example, multicistronic 2A sequence is used in a pCAGGS-K8.1-2A-gB-F-2A-gL-WT-2A-gH-F+HR2 vector or a pCAGGS-K8.1-2A-gB-F-2A-gL-WT-2A-gH-F-HR2 vector. The 2A sequence can be, for example, 15 amino acids, 16 amino acids, 17 amino acids, 18 amino acids, 19 amino acids, 20 amino acids, 21 amino acids, 22 amino acids, 23 amino acids, 24 amino acids, or 25 amino acids in length. In some embodiments, a sequence of GAAGAGA is used to generate fusion protein between the antigens. In some embodiments, a full-length NP sequence or a 26-amino acid of NP sequence is used to deliver or package the antigens into the VLPs.

[0024] The amino acid sequence of the full-length NP is as follows (SEQ ID NO:1):

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MSSVFDEYEQLLAAQTRPNGAHGGGEKGSTLKVEVPVFTLNSDDPEDRWNFVVFCLRIAV 60
SEDANKPLRQGALISLLCSHSQVMRNHVALAGKQNEATLAVLEIDGFTNSVPQFNNTSGV 120
SEERAQRFMMIAGSLPRACSNGTPTITAGVEDDAPEDIIDTLERILSIQAQVWTVAKAM 180
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TAYETADESETRINKYMQQGRVQKKYILHPVCRSAIQLTIRQSLAVRIFLVSELKRGRN 240
HAGGSSTYYNLVGDVDSYIRNTGLTAFFLTLYGINTKTSALALSSLAGDIQKMKQLMRL 300
YRMKGDNAPYMTLLGSDSQMSFAPAEYAQLYSFAMAMASVLDKGTGKYQFARDFMSTSF 360
RLGVEYAQAQGSSINEDMAAELKLTPAARRGLAAAAQRVSEETSSMDIPTQQAGVLTGLS 420
DGGPQAPQGGSNRSQGRPDAGDGETQFLDLGRAVANSRMREAPNSVQSTTQPEPPPTGPS 480
QDNDTDWGY 489

In some embodiments, the amino acid sequence of the 26 AA fragment of the NP is SVQSTTQPEPPPTGPSQDNDTDWGY (SEQ ID NO:2).

[0025] In some embodiments, disclosed herein is a method of producing KSHV-LPs with a polycistronic vector using one or more 2A sequences in a pCAGGS vector. The 2A sequence can be, for example, 15 amino acids, 16 amino acids, 17 amino acids, 18 amino acids, 19 amino acids, 20 amino acids, 21 amino acids, 22 amino acids, 23 amino acids, 24 amino acids, or 25 amino acids in length. The method can further entail generating fusion protein between the antigens or any other two or more virus latent proteins using a sequence of GAAGAGA (SEQ ID NO:3) to form a NP-fusion protein. In some embodiments, a full-length NP sequence or a 26-amino acid of NP sequence is used to deliver or package KSHV latent proteins into the VLPs.

[0026] In some embodiments disclosed herein, the KSHV glycoproteins can be expressed by any suitable expression vectors including plasmid vectors and viral vectors. In some embodiments, modified Ankara vaccinia vector, adeno-associated viruses, baculovirus or messenger RNA can be used for co-expressing two or more KSHV glycoproteins. The individual glycoproteins can be linked by cleavage sequences such that the co-expressed glycoproteins can be self-cleaved and self-assembled into two or more glycoprotein complexes.

[0027] In some embodiments, the expression systems or vectors described herein include two or more expression cassettes, each of which includes a single promoter and a sequence that encodes two or more KSHV glycoproteins. As a result, the two or more KSHV glycoproteins are co-expressed simultaneously, i.e., under control of a single promoter, obviating the need for multiple promoters or vectors. In certain embodiments, each expression cassette includes two, three, four, five, or even higher numbers of glycoproteins, the expression of which are under control of a single

promoter. In some embodiments, a vector may include more than one such expression cassette.

[0028] In some embodiments, 2A signal sequences that encode for the 2A peptide of food-and-mouth disease virus (F2A), equine rhinitis A virus (E2A), porcine teschovirus-1 (P2A), Thosaasigna virus (T2A), cytoplasmic polyhedrosis virus (BmCPV 2A), or flacherie virus (BmIFV 2A) can be used to link multiple genes under a single promoter. 2A signal sequences have been found in picornaviruses, insect viruses and type C rotaviruses. In some embodiments, a self-cleavage 2A peptide-derived sequence from Picornaviruses is used to co-express the KSHV glycoproteins. Bicistronic or multicistronic expression vectors can be used to express more than one gene product within a cell. Various suitable eukaryotic cell promoters can be used, including but not limited to, immediate-early I promoter of human CMV or the chicken beta actin promoter, promoters of vaccinia virus (mH5, pSyn, P11, p7.5), etc.

[0029] Additionally, a furin cleavage site preceding the 2A signal sequences can be incorporated to remove the 2A peptides following self-processing of the 2A-linked polyproteins. Furin is an enzyme that occurs in the Golgi apparatus and cleaves at very short signal peptides such as KKRR (SEQ ID NO:4) or RKRR (SEQ ID NO:5) motif. Furin cleavage contributes to protein processing and maturation. These short signal peptides can be added to the N-terminus of the 18-22 amino acid long 2A skipping signals so that they are removed following 2A-mediated processing of the KSHV envelope glycoproteins, except for one or two remaining amino acids. The resultant product can be even more "native." Although it is preferred that the 2A-linked glycoproteins are expressed all from one vector through the use of one or more expression cassettes, it is also possible to express the 2A-linked subunits from two or more separate vectors.

[0030] By exploiting the ribosomal skipping mechanism conferred by 2A peptides, an approach of co-expressing the KSHV glycoproteins as only one or two self-processing polyproteins is disclosed herein. The 2A ribosomal skipping system is widely-used to express multi-protein complexes due to the relative small sizes of 2A peptides (18-22 amino acids) and because it allows stoichiometric expression of the individual 2A-linked subunits. In some embodiments, P2A-linked DNA sequences of two or more KSHV glycoproteins are co-expressed and efficiently cleaved and transported to the cell surface. In some embodiments, the DNA sequences encoding

the KSHV glycoproteins are codon-optimized. In some embodiments, the co-expressed KSHV glycoproteins are self-assembled into surface complexes.

[0031] According to the embodiments described herein, an immunization regimen is provided. The immunization regimen includes VLPs comprising two or more KSHV glycoproteins and one or more T cell antigens. The immunization regimen may be administered via prime/boost homologous (e.g. using only the same vaccine type) or heterologous (e.g. using different vaccine types) vaccination. The immunization regimen may be administered in a dose vaccination schedule involving two or more immunizations, which may be administered 2 weeks to 6 months apart. Other suitable immunization schedules or regimens that are known in the art may be used according to the embodiments described herein by those skilled in the art.

[0032] The vaccine composition as described herein may comprise a therapeutically effective amount of a VLP as described herein, and may further comprise a pharmaceutically acceptable carrier according to a standard method. Examples of acceptable carriers include physiologically acceptable solutions, such as sterile saline and sterile buffered saline.

[0033] In some embodiments, the vaccine or pharmaceutical composition may be used in combination with a pharmaceutically effective amount of an adjuvant to enhance the anti-viral effects. Any immunologic adjuvant that may stimulate the immune system and increase the response to a vaccine, without having any specific antigenic effect itself may be used as the adjuvant. Many immunologic adjuvants mimic evolutionarily conserved molecules known as pathogen-associated molecular patterns (PAMPs) and are recognized by a set of immune receptors known as Toll-like Receptors (TLRs). Examples of adjuvants that may be used in accordance with the embodiments described herein include Alum, Freund's complete adjuvant, Freund's incomplete adjuvant, double stranded RNA (a TLR3 ligand), LPS, LPS analogs such as monophosphoryl lipid A (MPL) (a TLR4 ligand), flagellin (a TLR5 ligand), lipoproteins, lipopeptides, single stranded RNA, single stranded DNA, imidazoquinolin analogs (TLR7 and TLR8 ligands), CpG DNA (a TLR9 ligand), Ribi's adjuvant (monophosphoryl-lipid A/trehalose dicorynocolate), glycolipids (α -GalCer analogs), unmethylated CpG islands, oil emulsion, liposomes, virosomes, saponins (active fractions of saponin such as QS21), muramyl dipeptide, alum, aluminum hydroxide, squalene, BCG, cytokines such as GM-CSF and IL-12, chemokines such

as MIP 1- α and RANTES, activating cell surface ligands such as CD40L, N-acetylmuramine-L-alanyl-D-isoglutamine (MDP), and thymosin α 1. The amount of adjuvant used can be suitably selected according to the degree of symptoms, such as softening of the skin, pain, erythema, fever, headache, and muscular pain, which might be expressed as part of the immune response in humans or animals after the administration of this type of vaccine.

[0034] In further embodiments, use of various other adjuvants, drugs or additives with the vaccine of the invention, as discussed above, may enhance the therapeutic effect achieved by the administration of the vaccine or pharmaceutical composition. The pharmaceutically acceptable carrier may contain a trace amount of additives, such as substances that enhance the isotonicity and chemical stability. Such additives should be non-toxic to a human or other mammalian subjects in the dosage and concentration used, and examples thereof include buffers such as phosphoric acid, citric acid, succinic acid, acetic acid, and other organic acids, and salts thereof; antioxidants such as ascorbic acid; low molecular weight (e.g., less than about 10 residues) polypeptides (e.g., polyarginine and tripeptide) proteins (e.g., serum albumin, gelatin, and immunoglobulin); amino acids (e.g., glycine, glutamic acid, aspartic acid, and arginine); monosaccharides, disaccharides, and other carbohydrates (e.g., cellulose and derivatives thereof, glucose, mannose, and dextrin), chelating agents (e.g., EDTA); sugar alcohols (e.g., mannitol and sorbitol); counterions (e.g., sodium); nonionic surfactants (e.g., polysorbate and poloxamer); antibiotics; and PEG.

[0035] The vaccine or pharmaceutical composition containing the KSHV-LPs described herein may be stored as an aqueous solution or a lyophilized product in a unit or multiple dose container such as a sealed ampoule or a vial.

[0036] The expression systems, vectors and vaccines described herein may be used to treat or prevent a KSHV infection or conditions associated with KSHV infection such as an endothelial based Kaposi sarcoma, B cell-based lymphomas such as primary effusion lymphoma and multicentric Castleman's disease, carcinomas, and KSHV inflammatory cytokine syndrome.

[0037] As used herein, the term "subject" is an animal. In some embodiments, the subject is a mammal. In some embodiments, the subject is human.

[0038] The term “an effective amount” as used herein refers to an amount of a composition that produces a desired effect. For example, a population of cells may be infected with an effective amount of a viral vector to study its effect *in vitro* (e.g., cell culture) or to produce a desired therapeutic effect *ex vivo* or *in vitro*. An effective amount of a composition may be used to produce a prophylactic or therapeutic effect in a subject, such as preventing or treating a target condition, alleviating symptoms associated with the condition, or producing a desired physiological effect. In such a case, the effective amount of a composition is a “therapeutically effective amount,” “therapeutically effective concentration” or “therapeutically effective dose.” The precise effective amount or therapeutically effective amount is an amount of the composition that will yield the most effective results in terms of efficacy of treatment in a given subject or population of cells. This amount will vary depending upon a variety of factors, including but not limited to the characteristics of the composition (including activity, pharmacokinetics, pharmacodynamics, and bioavailability), the physiological condition of the subject (including age, sex, disease type and stage, general physical condition, responsiveness to a given dosage, and type of medication) or cells, the nature of the pharmaceutically acceptable carrier or carriers in the formulation, and the route of administration. Further an effective or therapeutically effective amount may vary depending on whether the composition is administered alone or in combination with another composition, drug, therapy or other therapeutic method or modality. One skilled in the clinical and pharmacological arts will be able to determine an effective amount or therapeutically effective amount through routine experimentation, namely by monitoring a cell's or subject's response to administration of a composition and adjusting the dosage accordingly. For additional guidance, see Remington: The Science and Practice of Pharmacy, 21st Edition, Univ. of Sciences in Philadelphia (USIP), Lippincott Williams & Wilkins, Philadelphia, PA, 2005, which is hereby incorporated by reference as if fully set forth herein.

[0039] “Treating” or “treatment” of a condition or an infection may refer to preventing the condition or the infection, slowing the onset or rate of development of the condition or the infection, reducing the risk of developing the condition or the infection, preventing or delaying the development of symptoms associated with the condition or the infection, reducing or ending symptoms associated with the condition or the infection, generating a complete or partial regression of the condition or the

infection, or some combination thereof. Treatment may also mean a prophylactic or preventative treatment of a condition or an infection.

[0040] In some embodiments, the vaccine or pharmaceutical composition described herein may be used in combination with other known vaccine or pharmaceutical products, such as immune response-promoting peptides, messenger RNAs, viral vectors such as modified vaccinia virus Ankara (MVA), adenovirus, baculovirus, and antibacterial agents (synthetic antibacterial agents). The vaccine or pharmaceutical composition may further comprise other drugs and additives. Examples of drugs or additives that may be used in conjunction with a vaccine or pharmaceutical composition described herein include drugs that aid intracellular uptake of the composition or vaccine disclosed herein, liposome and other drugs and/or additives that facilitate transfection, (e.g., fluorocarbon emulsifiers, cochleates, tubules, golden particles, biodegradable microspheres, and cationic polymers).

[0041] In some embodiments, the vaccine composition or pharmaceutical composition described herein may be administered by directly injecting a virus-like particle (VLP) suspension prepared by suspending the VLP in PBS (phosphate buffered saline) or saline into a local site, by nasal or respiratory inhalation, or by intravascular (i.v.) (e.g., intra-arterial, intravenous, and portal venous), subcutaneous (s.c.), intracutaneous (i.c.), intradermal (i.d.), or intraperitoneal (i.p.) administration. The vaccine or pharmaceutical composition disclosed herein may be administered more than once. More specifically, after the initial administration, one or more additional vaccinations may be given as a booster. One or more booster administrations can enhance the desired effect. After the administration of the vaccine or pharmaceutical composition, booster immunization with a pharmaceutical composition containing the VLP as described herein may be performed.

[0042] The following examples are provided to better illustrate the claimed invention and are not to be interpreted as limiting the scope of the invention. To the extent that specific materials are mentioned, it is merely for purposes of illustration and is not intended to limit the invention. It will be apparent to one skilled in the art that various equivalents, changes, and modifications may be made without departing from the scope of invention, and it is understood that such equivalent embodiments are to be included herein. Further, all references cited in the disclosure are hereby incorporated by reference in their entirety, as if fully set forth herein.

Example 1: Materials and Methods

[0043] Animals: Wild-type New Zealand white rabbits and BALB/c wild-type mice were purchased from Pocono Rabbit Farm and Laboratory Inc. and Jackson Laboratory, respectively. Rabbits and mice used for experiments were housed at Pocono Rabbit Farm and Laboratory Inc. and Beckman Research Institute of City of Hope, respectively. Animal procedures were performed in accordance with Pocono Rabbit Farm and Laboratory Inc. and Beckman Research Institute of City of Hope Institutional Animal Care and Use Committee and Institutional Biosafety Committee protocols.

[0044] Cells and virus: Chinese hamster ovary (CHO) cells, human embryonic kidney (HEK-293) cells, HEK-293 cells stably expressing Epstein-Barr virus nuclear protein 1 for enhanced ability to produce recombinant proteins (HEK-293 6E), human umbilical vein endothelial cells (HUVEC), human foreskin fibroblast 1 (HFF-1) cells, mouse myeloma cells (P3X63Ag8.653), and B cells derived from the pleural effusion of a patient with undifferentiated lymphoma (MC116) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Doxycycline-inducible iSLK cells harboring recombinant KSHV.219 expressing enhanced green fluorescent protein from the human elongation factor-1 α promoter (iSLD-rKSHV-eGFP.219) (35) were obtained from Dr. D. Dittmer of University of North Carolina, Chapel Hill, NC. CHO, HEK-293, HFF-1, and iSLK-rKSHV.219 cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM). P3X63Ag8.653 and MC116 cell lines were cultured in Roswell Park Memorial Institute medium 1640 (RPMI). Hybridomas were cultured in DMEM supplemented with nonessential amino acids (ThermoFisher, Waltham, MA). 1x oxaloacetate-pyruvate-insulin (MilliporeSigma, Burlington, MA), 4 mM glutamine, 1x hypoxanthine-aminopterin-thymidine (MilliporeSigma), 100 IU PS, 1x hybridoma cloning factor (MilliporeSigma), 55 μ M 2-mercaptoethanol, and 10% NCTC-109 (ThermoFisher Scientific). Media for culturing iSLK-rKSHV-eGFP.219 cells was supplemented with neomycin (250 μ g/mL), hygromycin (400 μ g/mL), and puromycin (10 μ g/mL) to maintain stable selection of both rKSHV-eGFP.219 and the RTA gene under the pRetro-X Tet-ON inducible system (35). All media were supplemented with 10% heat-inactivated fetal bovine serum (FBS) purchased from MilliporeSigma, 1% L-glutamine and 2% penicillin-streptomycin (ThermoFisher Scientific). HEK-293 6E cells were grown in Freestyle F17 Expression media without

FBS (ThermoFisher Scientific). HUVEC cells were grown in endothelial cell growth ready-to-use media (PromoCell, Edmonton, Alberta). Cell lines were routinely tested for mycoplasma using PCR and shown to be negative.

[0045] De-identified primary human tonsil specimens were obtained after routine tonsillectomy from the discarded de-identified tissues with approval from the Institutional Review Board of Chapman University. Lymphocytes were extracted by dissection and B cells were isolated and cryopreserved in 90% FBS and 10% DMSO as described (36). Cultured primary B cells were grown in complete RPMI.

[0046] To produce virus for infection and neutralization assays, iSLK-rKSHV-eGFP.219 cells were lytically induced and viruses were purified as described (37). In brief, iSLK-rKSHV-eGFP.219 cells were induced by the addition of 2 µg/ml doxycycline (Dox) and 1.5 mM sodium butyrate (NaB) to the culture media for four days and the harvested supernatant was clarified by centrifugation at 2,000 xg for 15 minutes at room temperature, followed by filtration through 0.8 µm membrane to remove cellular debris. Virions were pelleted by ultra-centrifugation (25% sucrose solution, SW41 rotor at 10,000 xg for 70 minutes at 4 °C). Purified viruses were quantified by titration in each of the cell types used in the infection and neutralization assays.

[0047] Antibodies: Primary mouse monoclonal immunoglobulin G2a (IgG2a) anti-gpK8.1 (clone 4A4) (38), which detects the ectodomain of the gpK8.1 protein, was purchased from Santa Cruz Biotechnology (Dallas, TX), and was used for fluorescence-activated cell sorting (FACS), immunoblot, and enzyme-linked immunosorbent assay (ELISA). Primary polyclonal rabbit anti-Newcastle disease virus (NDV) detecting nucleoprotein (NP) was a kind gift of Dr. T. Morrison, University of Massachusetts Medical School, Worcester, MA. Primary polyclonal goat anti-human IgG and rabbit anti-2A protein peptide were purchased from ThermoFisher Scientific and MilliporeSigma, respectively. Primary monoclonal antibodies anti-gpK8.1 (clone 41E7), and anti-gH/gL (clones 54A1 and 57C12) were generated and characterized as outlined below and used in immunoblot and ELISA. HRP-conjugated secondary antibodies goat anti-mouse IgG and goat anti-rabbit IgG, used for immunoblot and ELISA were purchased from MilliporeSigma. Goat Fab-2 anti-mouse IgG (H+L) cross-adsorbed secondary antibody, conjugated to Alexa Fluor 488 (AF488), was purchased from ThermoFisher Scientific and used for FACS.

[0048] Construction of plasmids to generate KSHV-LPs: To generate polyvalent KSHV-LP vaccine candidates incorporating multiple KSHV proteins (gpK8.1, gB, gL and gH), two cDNAs were constructed and synthesized (Genewiz, South Plainfield, NJ). Each sequence included the gpK8.1 ectodomain (ED) fused to NDV fusion protein (F) transmembrane /cytoplasmic (TM/CT) domains (gpK8.1-F); the gB ED fused to NDV F TM/CT (gB-F), full-length wild-type gL without an F fusion (WTgL); and the gH ED fused to NDV F TM/CT (gH-F). In one cDNA, each NDV F TM/CT domain also contained heptad region 2; in the other cDNA, it did not (+/-HR2). To express this complex in its native form, unique 2A linker sequences (18 amino acids long) from picorna virus (41) were interspersed between individual glycoprotein cDNAs. See Table 1.

Table 1. 2A Linker Sequences		
2A Position	Nucleotide Sequence	Amino Acid Sequence
gpK8.1-gB	GCTACTAACTTCAGCCTGCTGAAGCAGGCT GGAGACGTGGAGGAGAACCCTGGACCT (SEQ ID NO:6)	ATNFSLLKQAGDV EENPGP (SEQ ID NO:7)
gB-gL	GCGACTAACTTCTCATTGTTGAAACAGGCA GGAGATGTCTGAAGAGAACCCTGGTCCA (SEQ ID NO:8)	ATNFSLLKQAGDV EENPGP (SEQ ID NO:9)
gL-gH	GCAACGAATTTCTCCCTTCTAAAGCAAGCC GGTGACGTGGAGGAGAATCCCGGACCC (SEQ ID NO:10)	ATNFSLLKQAGDV EENPGP (SEQ ID NO:11)

[0049] As disclosed herein, use of 2A linkers resulted in a polycistronic plasmid with cleavage sites that allow gpK8.1-F, gB-F, WTgL, and gH-F to be processed independently after transcription, and released to natively incorporate on the VLP envelope. The two synthesized chimeric cDNAs (gpK8.1-F-2A-gB-F 2A-WTgL-2A-gH-F+/-HR2) cloned into a mammalian expression plasmid, pCAGGS. Plasmids encoding for full-length NDV NP and M cloned into a pCAGGS plasmid have been described. Sanger sequencing was used to verify the sequence fidelity of all synthesized and cloned constructs. All primer sequences used in the study are listed in Table 2.

Table 2. Primer Sequences	
Primer Name	Primer Sequence (5'-3')
Cloning Primers	
KSHV K8.1-Fc-His FWD	AAAAAGCGGCCGCGCCACCATGAGTTCCACACAGATTCTG (SEQ ID NO:12)
KSHV K8.1-Fc-His REV	AAAAAACTAGTGTAAGATGGGTCCGTATTTCTGC (SEQ ID NO:13)
KSHV gB-Fc-His FWD	AAAAAGCGGCCGCGCCACCATGACTCCCAGGTCTAGATTGG (SEQ ID NO:14)
KSHV gB-Fc-His Rev	AAAAAACTAGTTTTAATAAAATTTATGAATCCGGTAAC (SEQ ID NO:15)
KSHV gH-gL Gibson.FOR	TTCTCGAGGATCCGCGGCCGCGCCACCATGGGGATCTTTGCGCTA TTTG (SEQ ID NO:16)
KSHV gHgL Gibson.REV	GCATGTGTGAGTTTTGTCAGTGTGCGCGTCTTCTATACATGCC (SEQ ID NO:17)
Sequencing Primers	
FC-Hisseqprimer1	GTCGAGGTCTCGACGGTATCG (SEQ ID NO:18)
FC-Hisseqprimer2	CTCTATAGGCACACCCCTTTGG (SEQ ID NO:19)
FC-Hisseqprimer3	CGCGCGCCACCAGACATAATAG (SEQ ID NO:20)
FC-Hisseqprimer4	GCTTTAATAAGATCTCTAG (SEQ ID NO:21)
FC-Hisseqprimer5	TGCTGGGCACGGTGGGCATG (SEQ ID NO:22)
FC-Hisseqprimer6	GGGTCTTTTCTGCAGAAGCTTG (SEQ ID NO:23)
FC-Hisseqprimer7	CTGACCAAGAACCAGGTCAGC (SEQ ID NO:24)
FC-Hisseqprimer8	GACGCTCAAGTCAGAGGTGGC (SEQ ID NO:25)
FC-Hisseqprimer9	CTCCCCGTCGTGTAGATAACTAC (SEQ ID NO:26)
FC-Hisseqprimer10	CAATATTATTGAAGCATTTATC (SEQ ID NO:27)
FC-Hisseqprimer11	TGCGTAAGGAGAAAATACCGC (SEQ ID NO:28)

[0050] Generation and production of MVA-KSHV-LPs: For MVA-KSHV-5Ag-VLP construct, vectors for gene insertion into the Deletion III site (Del3) (mH5-M), and the insertion site between the essential ORFs G1L and I8R a 2A-linked polycistronic

expressing transcript including the K8.1 ectodomain (ED) fused to NDV fusion protein (F) transmembrane/cytoplasmic (TM/CT) domains (K8.1-F); the gB ED fused to NDV F TM/CT (gB-F); and the gH ED fused to NDV F TM/CT (gH-F) without heptad region 2 (-HR2); and full-length wild-type gL (WTgL) between gB and gH, inserted into the MVABAC-TK can be transferred. Additionally, the IGR3 site inserted with NDV NP (28 amino acids) fused with partial LANA1 (937-1124 amino acids) protein of KSHV, as depicted in Figure 5.

[0051] Transfection and generation of stable cells to produce KSHV-LPs:

To generate gpK8.1-gB-gL-gH KSHV-LPs, 2 µg of pCAGGS-gpK8.1-F-2A-gB-F-2A-WTgL-2A-gH-F+/-HR2 plasmid was transiently transfected into CHO or HEK-293 cells using linear polyethylenimine (PEI) transfection reagent (ThermoFisher Scientific). After 48 hours, cells were stained with anti-K8.1 (clone 4A4) primary antibody for 30 minutes, washed three times with 1x phosphate buffered saline (PBS), and stained with goat anti-mouse secondary antibody conjugated to AF488. Stained cells were washed three times and analyzed using FACS to detect surface expression of gpK8.1 on the cells. Untransfected and unstained CHO or HEK-293 cells served as negative controls for FACS analysis. Upon confirmation of gpK8.1 expression in both CHO and HEK-293 cells, only CHO cells were then co-transfected as above with individual pCAGGS-gpK8.1-F-2A-gB-F-2A-WTgL-2A-gH-F+/-HR2 and pCI-puro plasmids, followed by 5 µg/µl of puromycin selection 48 hours post-transfection, to generate stable cells expressing the four glycoproteins. Stable CHO cells resistant to puromycin selection were pooled and stained with primary anti-gpK8.1 mAb (4A4), followed by secondary goat Fab-2 anti-mouse IgG (H+L) conjugated to AF488 and sorted five times to generate a colony of CHO cells expressing mainly gpK8.1-gB-WTgL-gH+/-HR2 (~50% anti-K8.1-AF488 positive). Stable cells were then co-transfected with equal amounts of both pCAGGS-NDV M and pCAGGS-NDV NP plasmids to produce gpK8.1-gB-gH/gL+/-HR2 VLPs (KSHV-LPs). Additional VLPs were also produced by transiently co-transfecting CHO or HEK-293 cells with pCAGGS encoding for NDV M, NDV NP, and polycistronic gpK8.1-F-2A-gB-F-2A-WTgL-2A-gH-F+/-HR2 plasmids.

[0052] Purification and characterization of KSHV-LPs: Supernatants from stable cells releasing KSHV-LPs were collected 24-120 hours post-transfection, and combined, then cell debris was removed by centrifugation at 3,724 xg (Corning 500 ml centrifuge tubes, SX4750 rotor) for 15 minutes at 4°C. To pellet the KSHV-LPs, the

resultant supernatant was centrifuged in a type 19 fixed angle aluminum rotor (Beckman Coulter, Brea, CA) at 9,846 xg at 4°C for 12 hours. The resultant pellet was re-suspended in 4 ml TNE buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 5 mM EDTA) and layered on a discontinuous sucrose gradient composed of 2 ml of 65% sucrose and 4 ml of 20% sucrose. The gradients were centrifuged at 42,953 xg at 4°C in an SW 40Ti rotor (Beckman Coulter, Brea, CA) for 12 hours. The fluffy layers at the 20-65% interface containing the KSHV-LPs were collected, mixed with two volumes of 80% sucrose and sandwiched between 1 ml of 80% sucrose (at the bottom of the tube) and 3.5 ml of 50% sucrose (on top); 2 ml of 10% sucrose was then laid on top. The mixture was centrifuged for 12 hours at 91,349 xg in a type SW 40Ti rotor at 4°C. The resultant VLP fluffy layer was collected, re-suspended in 1x TNE and pelleted at 91,349 xg in an SW 40Ti rotor (Beckman) at 4°C for 1 hour. The resultant pellet was then re-suspended in TNE, total protein was quantified using BCA assay (Pierce, Rockford, IL) and purified VLPs were stored at -20°C or 4°C until further use.

[0053] Construction, purification and characterization of KSHV gpK8.1, gB and gH/gL proteins: To construct KSHV gpK8.1, gB, and gH/gL Fc-6xHis tagged plasmids, the coding sequence for each protein ED was PCR-amplified with gene-specific primers (Table 1) from previously described pCAGGS gpK8.1, pCAGGS gB, and pCAGGS gH/gL constructs. The upstream (5') and downstream (3') primers contained NotI and SpeI enzyme restriction sites (gpK8.1 and gB), respectively, or Gibson assembly primers (gH/gL), which were used to subclone the PCR products into the Cntn1-Fc-His plasmid from Addgene plasmid #72065 (Watertown, MA), a gift from Dr. W. Wojtowicz, Stanford University, Palo Alto, CA.

[0054] Fc-6xHis-tagged recombinant KSHV gpK8.1, gB and gH/gL proteins were expressed via transient transfection of HEK-293 6E using linear PEI transfection reagent. Cells were seeded a day before transfection at 800,000 cells/ml in a 250 ml of media in a 1-liter Erlenmeyer flask, the following day 200 µg of plasmid DNA and linear PEI at a 1:5 DNA:PEI ratio were added to 9 ml Opti-MEM (ThermoFisher Scientific), mixed gently, incubated for exactly 20 minutes at room temperature and added dropwise onto cells to make a final volume of 250 ml. Culture media was harvested six days post-transfection by centrifugation and filtration through a 22 µm filter. The Fc-6XHis-tagged KSHV proteins in the media were purified using Protein A spin columns (Takara Bio, Kusatsu, Shiga, Japan), buffer exchanged and

concentrated into PBS using Amicon Ultra 15 centrifugal filter units (MilliporeSigma) and then quantified using a nanodrop spectrophotometer (ThermoFisher Scientific).

[0055] Generation and identification of KSHV-glycoprotein-specific monoclonal antibodies: Monoclonal antibodies specific to KSHV gpK8.1, and gH/gL were generated using standard techniques (44). Briefly, to generate, identify, and characterize KSHV-glycoprotein-specific mAbs, four wild-type BALB/c mice were immunized intraperitoneally with 200 μ l containing 100 μ g of complete Freund's adjuvant (MilliporeSigma F5881) mixed with 50 μ g of purified UV-inactivated rKSHV-eGFP.219 (UV-KSHV) resuspended in TNE buffer. The animals were boosted five times with 200 μ l containing 100 μ g of incomplete Freund's adjuvant (MilliporeSigma F5506) and 25 μ g of UV-KSHV resuspended in TNE buffer every two weeks. Once optimal antibody response to gpK8.1 and gH/gL in sera was confirmed using ELISA, a sixth booster immunization was provided intravenously with 200 μ l containing 25 μ g of UV-KSHV resuspended in TNE. Boosted mice were sacrificed, and splenocytes were isolated and fused with a mouse myeloma cell line (P3X63Ag8.653) to generate murine hybridomas, using a standard polyethylene glycol (PEG) method, and cultured as described (44). On Day 7 post-fusion, cells were fed with 100 μ l hypoxanthine-aminopterin-thymidine medium media per well. On Days 10–14, growing hybridomas were resuspended and cells reseeded in duplicate 96-well plates and allowed to grow for three days. Supernatants from one of the duplicate 96-well plate hybridoma plates were collected to perform indirect ELISA with purified KSHV proteins (gpK8.1 or gH/gL) to identify hybridoma colonies that secreted murine IgG antibodies specific to KSHV gpK8.1 or gH/gL. Positive hybridomas were expanded from the second 96-well plate and screened by performing confirmatory ELISA, FACS and immunoblot. Identified antibodies were expanded and bulk purified through protein A spin columns and stored at -20°C for subsequent assays.

[0056] Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), Coomassie staining and immunoblotting: Cells and KSHV-LPs were lysed in RIPA buffer (10 mM Tris-HCL, pH 8.0, 1 mM EDTA, 140 mM NaCl, 0.1 Triton X-100, protease inhibitor cocktail) (MilliporeSigma). The lysed suspensions were vigorously vortexed, incubated on ice for 30 minutes, and then centrifuged at 15,871 xg for 10 minutes at 4°C in a benchtop centrifuge. The supernatants were collected and total protein was quantified using BCA assay. A known quantity of protein was

loaded onto a 4-12% polyacrylamide gel for protein separation using 1x MES SDS running buffer (ThermoFisher Scientific), then either the gel was stained with Coomassie blue or proteins were transferred from the gel to a polyvinylidene fluoride membrane using iBlot (ThermoFisher Scientific) for immunoblot analysis. Immunoblot analyses for proteins (i.e., gpK8.1, gB, gH/gL, and NDV-NP) were performed by blocking the membrane with 3% bovine serum albumin (MilliporeSigma) in Tris-buffered saline (TBS) for 1 hour at room temperature, followed by detection with relevant specific primary and secondary antibodies as described (30). 2A protein peptide and Fc-tagged proteins were detected using their respective antibodies following antibody manufacturers' protocols.

[0057] Transmission electron microscopy (TEM): To assess the composition of KSHV-LPs compared to wild-type KSHV, morphological examination was conducted using TEM. KSHV-LPs were purified as described above and rKSHV-eGFP.219 virus was harvested and purified from iSLK KSHV-eGFP.219 cells as described. Purified KSHV-LPs+/- HR2 and rKSHV-eGFP.219 were fixed in 4% paraformaldehyde in preparation for TEM. Specimens were absorbed to glow-discharged, carbon-coated, 200-mesh EM grids. Samples were prepared by conventional negative staining with 1% (w/v) uranyl acetate. EM images were collected with an FEI Tecnai 12 TEM (ThermoFisher Scientific) equipped with a LaB6 filament and operated at an acceleration voltage of 120 kV. Images were recorded with a Gatan 2x 2 k CCD camera (Gatan, Inc., Pleasanton, CA, USA) at a magnification of 21,000X and a defocus value of ~1.5 μm .

[0058] Immunization of wild-type New Zealand white rabbits and collection and processing of serum samples: Female and male wild-type New Zealand white rabbits 8-10 weeks old were immunized subcutaneously three times on Days 0, 21, and 42 with 50 μg total protein of purified individual KSHV-LPs+/-HR2 (n=6 each group) in 0.5 ml TNE buffer adsorbed to 500 μg aluminum hydroxide (alum) mixed with 50 μg of monophosphoryl lipid A from *Salmonella enterica* serotype Minnesota Re595 (MPL) as adjuvants; alum/MPL-adjuvanted UV-KSHV (n=6) or alum/MPL-adjuvanted TNE (n=6) served as positive and negative controls, respectively. Rabbits were bled before immunization (pre-bleed baseline) and on Days 14, 35, 49, 70, and 90 (terminal bleed).

[0059] Following collection of blood, whole blood was allowed to coagulate for 2 hours at room temperature, then incubated overnight at 4 °C. To process whole blood into serum, the whole blood was centrifuged at 2,348 xg for 15 minutes at 4 °C. The serum was transferred into a clean tube and centrifuged again at 2,348 xg for 5 minutes at 4 °C. The final serum was poured into a vial and then crimp-sealed. The vial of serum was then stored at -80°C until being aliquoted for subsequent experiments.

[0060] Measurement of anti-gpK8.1-, gB- and gH/gL-specific antibody titers in sera from immunized rabbits using ELISA: Using sera collected from wild-type New Zealand white rabbits, antibodies titers were assessed by ELISA, using individual purified glycoproteins as the target. Briefly, wells of 96-well microtiter plates were coated with 0.5 µg of individual purified glycoprotein EDs (KSHV gpK8.1, gB, or gH/gL) in 50 µl of PBS buffer at 4°C overnight, and blocked with 3% BSA in 1x PBS and 0.1% tween for 1 hour at room temperature. Serially diluted sera (1:100, 1:300, 1:900, 1:2,700 and 1:8,100 in PBS) were added to the protein coated plates in quadruplicates and incubated for 2 hours at room temperature. Plates were washed three times and antibody binding was detected using HRP-labeled goat anti-rabbit IgG secondary antibodies for 1 hour at room temperature. Plates were washed three times, the substrate tetramethylbenzidine (ThermoFisher Scientific) was added for 20 minutes at room temperature, then the reaction was stopped with 1x KPL ABTS peroxidase stop solution (Sera Care, Milford, MA). To determine antibody titer, optical density was read at 405 nm using an ELISA reader, FilterMax F3 (Molecular Devices, San Jose). The highest antibody dilution yielding an OD₄₀₅ two times higher than that of TNE-treated rabbits (negative control) was designated as the endpoint titer.

[0061] Purification of IgG antibodies from rabbit sera and determination of anti-gpK8.1-, gB-, and gH/gL-specific antibodies: Purification of IgG antibodies was conducted by pooling Day 49 sera (highest antibody titer) from immunized rabbits (n=6) from each of the four treatment/control groups. Pooled sera were diluted 1:10 in equilibration/binding/wash buffer (1.5M glycine, 3M NaCl, pH 9.0), added to protein A columns, and centrifuged at 2,000 xg for 3 minutes at room temperature. Centrifugation flow-through was discarded and the columns were washed with 6 ml of wash buffer, followed by 1 ml of elution buffer (0.2 M Glycine, pH 2.5); samples were eluted into new collection tubes containing 100 µl neutralization buffer (1M Tris/HCl,

pH 9.0) by centrifugation at 2,000 xg for 3 minutes at room temperature. Purified IgGs were buffer exchanged and concentrated in PBS, protein concentration was determined using nanodrop, and IgGs were separated using SDS-PAGE and stained with Coomassie to determine purity. ELISA was used to determine the titers of anti-gpK8.1-, gB-, and gH/gL-specific antibodies from purified IgGs at different concentrations (25, 12.5, 6.25, 3.125, and 1.56 µg/ml). IgGs were stored at 4°C until use in subsequent experiments.

[0062] *In vitro* neutralization assay: *In vitro* neutralization was performed using different cell types of human origin, namely epithelial (HEK-293), fibroblast (HFF-1), endothelial (HUVEC), and B (MC116) cells. To conduct neutralization, rKSHV-eGFP.219 was first tittered in individual cell types. Briefly, each individual cell line was seeded in quadruplicate at a density of 5×10^5 in 48-well-plates and cultured overnight. The individual cell lines were then incubated with 5, 10, 20, 30, or 50 µl of the purified virus in a total volume of 100 µl of virus plus serum-free media for 24 hours at 37°C. Infected cells (eGFP-positive) were quantified using FACS by acquiring a total of 10,000 events and analyzed using FlowJo Software (FlowJo LLC, Ashland, Oregon) as described.

[0063] Upon determination of virus titer, cells were seeded as above. Purified IgGs were serially diluted in serum-free media to 12.5, 25, 50, or 100 µg/ml and incubated for 1 hour at 37°C with a known quantity of the virus that yielded 20-30% infectivity in the respective cell type used. The mixture of purified IgGs and virus were then added to the seeded cells and incubated for 2 hours at 37°C for adherent cells or spinoculated at 233 xg for 30 minutes at room temperature for B cells (MC116). Infected cells were washed three times in 1x PBS to remove excess IgGs and circulating viruses, and complete media were added and cells were incubated for 24 hours at 37°C. The number of eGFP-positive cells (i.e., rKSHV-eGFP.219-infected cells) was enumerated using FACS by acquiring 10,000 events. All dilutions were performed in quadruplicate and assays repeated at least once.

[0064] Statistical analysis: Statistical analyses were performed using GraphPad Prism 8 Software (GraphPad Software, San Diego, CA, USA). Multiple comparisons between groups were calculated using Kruskal-Wallis, whereas differences between groups were computed using Dunn's post hoc test and Mann-Whitney U test. All statistical tests were two-tailed, and a p-value equal to or lower

than 0.05 was considered significant. IC_{50} ($\mu\text{g/ml}$) values were calculated from neutralization data of KSHV.219 by purified IgGs in HEK-293, HFF-1, HUVEC and MC116 cells using nonlinear, dose-response regression analysis.

Example 2: Construction, purification, and characterization of KSHV-LPs that incorporate four KSHV proteins as vaccine candidates

[0065] It was previously demonstrated that the NDV-LP platform could be used to generate iKSHV-LPs incorporating a single surface envelope glycoprotein (gpK8.1 or gB) or two-glycoprotein complex (gH/gL) (30). In this study, two versions of a single polycistronic plasmid encoding sequences for four glycoproteins, designated pCAGGS-gpK8.1-F-2A-gB-F-2A-WTgL-2A-gH-F+/-HR2, as reagents for making multivalent KSHV-LPs (Figure 1A). In these plasmid constructs, the EDs of three KSHV glycoproteins (gpK8.1, gB, and gH) were fused to the structural protein NDV F with or without HR2; the NDV F HR2 domain was used to support trimerization of the proteins and help increase the number of immunogens per VLP. Full-length wild-type KSHV gL was incorporated because it does not have a transmembrane domain and depends on gH for its transport to the surface of the virion. All four glycoproteins were interspersed with a picornavirus 2A self-cleaving peptide for efficient expression and cleavage under a single cytomegalovirus promoter.

[0066] To confirm plasmid DNA transfection efficiency and expression of various glycoproteins from the polycistronic plasmids, each plasmid was transiently transfected into CHO or HEK-293 cells, two epithelial cell lines widely used in most laboratories for protein expression. To confirm the expression of proteins on the cell surface, transfected cells were stained with anti-K8.1 primary antibody, followed by mouse secondary antibody conjugated to AF 488, then FACS was used to detect surface expression of gpK8.1. The previously constructed pCAGGS-K8.1-F+HR2 plasmid was used as a positive control for gpK8.1 expression and untransfected cells and unstained transfected cells were used as negative controls. Transfected CHO and HEK-293 cells expressed gpK8.1 (Figure 1B). However, the total number of transfected cells expressing gpK8.1 was low in both HEK-293 and CHO cells transfected with the polycistronic plasmids compared to cells transfected with the plasmid encoding for a single protein. For example, in CHO cells transfected with gpK8.1-F-2A-gB-F-2A-WTgL-2A-gH-F -HR2 or +HR2, gpK8.1 expression was found to

be 4.74% and 6.77%, respectively, compared to 31.8% in CHO cells transfected with pCAGGS-gpK8.1-F+HR2. This occurred despite the optimization of transfection efficiency using various ratios of DNA:PEI or other transfection reagents (data not shown), and was probably due to the large cDNAs cloned into the plasmids.

[0067] To overcome poor transfection efficiency and improve KSHV-LP production for full characterization and immunogenicity testing, CHO cells stably expressing each multicistronic plasmid were established. Only CHO cells were used because over two-thirds of recombinant therapeutic agents on the market are generated in CHO cell lines and because no adventitious human pathogen has been reported for CHO cells. CHO cells were co-transfected with pCAGGS-gpK8.1-F-2A-gB-F-2A-WTgL-2A-gH-F+/-HR2 and pCI-puro. The transfected cells were cultured in 5 µg/ml of puromycin, resistant cells were stained with anti-K8.1-AF488 antibody and FACS sorted five times to enrich for cells expressing gpK8.1 (Figure 2A). Selection was based on gpK8.1 due to a lack of well-validated monoclonal antibodies against the other KSHV glycoproteins at the time of making the stable cell lines.

[0068] To produce KSHV-LPs, enriched stable cells were co-transfected with both pCAGGS-NDV M and pCAGGS-NDV NP, and then supernatants from the transfected cells were collected daily from 24 hours post-transfection until 120 hours post-transfection. KSHV-LPs were purified from the supernatants using sucrose-gradient sedimentation, followed by floatation gradients (Figure 2B), then fully characterized for their composition and structure as described (30). In brief, immunoblotting was used to confirm that all four KSHV glycoprotein subunits were incorporated into the KSHV-LPs+/-HR2. Rabbit polyclonal anti-2A and anti-NDV and mouse monoclonal anti-K8.1 and anti-gH/gL antibodies were used to show that all components (i.e., KSHV glycoproteins and NDV structural proteins) were incorporated and packaged into VLPs (Figure 2C). The use of anti-2A also detected unidentified proteins of higher molecular weights in VLPs; whether these are uncleaved proteins incorporated in the VLPs or cell debris in the purified VLP fraction remains to be elucidated. Because the 2A sequence between WTgL and gH-F is cleaved and remains with WTgL, it is not possible to use the anti-2A antibody to detect gH. Thus, to further confirm the specificity of the K8.1 protein bands and also detect gH/gL protein complex, specific monoclonal antibodies anti-gpK8.1 (41E7), and anti-gH/gL (57C12) generated in the laboratory were used to detect the individual proteins (Figure

2C). No commercially available KSHV anti-gB antibody can be used to prove the presence of gB in the KSHV-LPs. However, the molecular weight identified for each KSHV subunit agreed with published data, providing strong evidence that KSHV-LPs generated from a polycistronic plasmid are faithful to the composition of the subunits. Electron microscopy was used to confirm that the size, shape, and morphology of KSHV-LPs were similar to rKSHV-eGFP.219 virus: the VLP diameters were ~100 nm, with a spherical shape and spike-like structures, resembling glycoproteins incorporated into the virions (Figure 2D).

Example 3: Rabbits immunized with KSHV-LPs+/-HR2 elicited high titer KSHV-glycoprotein-specific antibody responses

[0069] The results of the previous experiments demonstrated that mice immunized singly or with combinations of KSHV-LPs expressing gpK8.1, gB, or gH/gL without adjuvant were able to generate robust antibody responses and stimulate KSHV glycoprotein-specific immune recall upon boosting (30). To demonstrate the ability of the multivalent KSHV-LPs disclosed herein to induce KSHV subunit-specific antibody responses, wild-type New Zealand white rabbits were immunized with alum/MPL-adjuvanted KSHV-LPs +/-HR2, and then ELISA was used to determine antibody titers and kinetics over time. Selection of the alum and MPL formulation was based on AS04, an adjuvant licensed for human use that is currently being used in two licensed VLP-based vaccines, the human papillomavirus vaccine Cervarix® (GlaxoSmithKline) and the hepatitis B vaccine FENDrix® (GlaxoSmithKline). Because there is no good standard for measuring IgGs against KSHV infection in human or immunized animals due to the inconsistent detection of KSHV-specific protein IgGs in KSHV-infected individuals, purified proteins were used as binding targets for use in ELISA as described (45). Taking advantage of the constructed plasmids individually encoding for soluble gpK8.1, gB, or gH/gL proteins, each plasmid was transfected into HEK-293 6E cells (which enabled high-yield production of post-translationally modified recombinant proteins in serum-free media), supernatants from the transfected cells were collected, clarified and purified through protein A columns. The purified proteins were concentrated and quantified and their authenticity was confirmed by both Coomassie blue staining and immunoblot using specific anti-gpK8.1 or anti-gH/gL monoclonal antibodies or polyclonal anti-human Fc for gB (Figure 3A).

[0070] To characterize antibody immune responses, 8–10-week-old wild-type New Zealand white rabbits were immunized subcutaneously three times (Days 0, 21, and 42) with 50 µg of purified KSHV-LPs +/-HR2. Each of the KSHV-LPs was suspended in 0.5 ml TNE buffer adsorbed to 500 µg alum and 50 µg MPL. The alum and MPL adjuvants were used to improve humoral and cellular immunogenicity. Rabbits immunized with 50 µg of purified UV-KSHV or TNE, adjuvanted with alum/MPL, served as positive and negative controls, respectively. To obtain enough sera to support complete characterization of antibody responses, 6 rabbits per group were used. To assess short- and long-term antibody responses, rabbits were bled to obtain sera seven days before the first immunization (pre-bleed) and on Days 14, 28, 35, 49, 70, and 90 (terminal bleed) (Figure 3B). ELISA was used to determine KSHV-glycoprotein-specific antibody titers from each individual animal over time using the purified proteins described above as the binding targets. All rabbits immunized with KSHV-LPs +/-HR2 or UV-KSHV generated KSHV-glycoprotein-specific IgG antibody responses that increased following booster immunizations, compared to TNE-immunized rabbits or pre-bleeds (negative controls) (Figure 3C). The increase in KSHV glycoprotein-specific antibody response peaked by Day 49, after the second booster immunization. There was no difference in the titers of the glycoproteins (K8.1, gB, or gH/gL) between rabbits immunized with KSHV-LPs +HR2 or -HR2. Compared to either KSHV-LP +/-HR2, UV-KSHV immunization elicited significantly higher titers of KSHV-glycoprotein-specific antibodies, which remained high through Day 90. In contrast, the antibody titers from animals immunized with either KSHV-LP +/-HR2 dropped by Day 70 (Mann-Whitney test; gpK8.1 Day 49 vs Day 70: KSHV-LPs -HR2 p=0.0022, KSHV-LPs +HR2 p=0.0022; gB Day 49 vs Day 70: KSHV-LPs -HR2 p=0.0022, KSHV-LPs +HR2 p= 0.0022; gH/gL Day 49 vs Day 70: KSHV-LPs -HR2 p=0.0043, KSHV-LPs +HR2 p=0.0303), suggesting that long-term antibody responses in KSHV-LP +/-HR2-immunized rabbits were weaker than those of UV-KSHV immunized rabbits.

Example 4: KSHV-LPs +/-HR2 elicit robust neutralizing antibodies that prevent rKSHV-eGFP.219 infection in diverse cell types

[0071] To measure the ability of KSHV-glycoprotein-specific polyclonal antibodies in sera from rabbits immunized with multivalent KSHV-LPs to neutralize

viral infection and to reduce the previously observed serum effect, IgGs from Day 49 samples (highest titers) were pooled and purified, and then KSHV-glycoprotein-specific IgGs were quantified using ELISA (Figure 4A). Similar to unpurified sera, titers for gpK8.1-, gB-, and gH/gL-specific IgGs were significantly higher in rabbits immunized with purified KSHV-LPs+/-HR2 compared to TNE (negative control) using a non-parametric Kruskal-Wallis test (gpK8.1, $p=0.0003$; gB, $p=0.0002$; gH/gL, $p=0.0012$). KSHV-specific IgG titers were higher in rabbits immunized with KSHV-LPs-HR2 than KSHV-LPs+HR2; for example, at 25 $\mu\text{g/ml}$, gpK8.1, gB and gH/gL IgG titers were 4-fold higher. However, titers were highest in rabbits immunized with UV-KSHV. For example, at 25 $\mu\text{g/ml}$, gpK8.1, gB and gH/gL IgG titers in UV-KSHV were 3-fold and 10-fold higher than in KSHV-LPs-HR2 and KSHV-LPs+HR2 IgG-purified samples, respectively (Figure 4A).

[0072] After confirming the presence and titers of KSHV-glycoprotein-specific antibodies in the purified sera, neutralization activity assays were performed in diverse cell types that represented most of the cell types permissive to KSHV infection *in vivo*. First, purified rKSHV-eGFP.219 was titrated in four permissive cell types of human origin to determine volumes that ensured >20% rKSHV-eGFP.219 infectivity for *in vitro* neutralization (Figure 4B, Panel i). To conduct *in vitro* neutralization assays, serially diluted purified IgGs (12.5, 25, 50, or 100 $\mu\text{g/ml}$) were incubated with rKSHV-eGFP.219. The percentage of eGFP+ cells (i.e., KSHV-infected cells) was measured using FACS and an effective neutralization titer was defined as 50% inhibition of infection, after normalization to control IgG samples from TNE-immunized rabbits. When rKSHV-eGFP.219 virus was pre-incubated with serially diluted concentrations of total purified IgG antibodies (12.5, 25, 50, or 100 $\mu\text{g/ml}$) from TNE-immunized rabbits (negative control), all concentrations achieved ~8% neutralizing activity (serum effect) in a non-dose-dependent manner in HEK-293 cells (data not shown). Thus, 8% was used as the normalization percentage to account for the serum effect in all subsequent neutralization assays. In contrast, rKSHV-eGFP.219 pre-incubated with serially diluted concentrations of pooled purified IgGs from rabbits immunized with KSHV-LPs +/-HR2 or UV-KSHV resulted in a dose-dependent reduction of KSHV infection in an endothelial cell line (HUVEC), foreskin fibroblast cell line (HFF-1), and B cell line (MC116) (Figure 4B, Panel ii). Purified IgGs from both groups of KSHV-LP +/-HR2-immunized rabbits blocked rKSHV-eGFP.219 infection *in vitro* in all cell types

tested (Figure 4B, Panel ii). Interestingly, despite UV-KSHV-immunized rabbits having higher titers of KSHV-glycoprotein-specific IgGs than KSHV-LP-immunized rabbits (Figure 4A), there were no statistical differences in the neutralizing antibody activities between them in all cell lines tested (Figure 4B). At the highest dilution analyzed (100 µg/ml), UV-KSHV and both KSHV-LPs +/-HR2 neutralized KSHV infection by 60–90% in all cell types tested (Figure 4B, Panel ii). Importantly, even at the lowest concentration, IgGs from any of the vaccine candidates neutralized KSHV infection by 12–55%, in all cell types tested, with the highest neutralization activity being observed in HFF-1 cells. The calculated IC₅₀ (Table 3) varied between cell lines and vaccine candidates: UV-KSHV, 14.07–28.89 µg/ml; KSHV-LPs -HR2, 26.56–37.97 µg/ml; and KSHV-LPs +HR2, 29.57–49.64 µg/ml. The results confirmed that a select group of KSHV glycoproteins incorporated on the surface of VLPs, and administered with appropriate adjuvants, can stimulate neutralizing antibodies that block KSHV infection of diverse cell types *in vitro*.

Table 3 IC₅₀ (µg/ml)			
Cell-line	UV-KSHV	KSHV-LPs –HR2	KSHV-LPs+HR2
HEK-293	27.36	33.49	39.9
HFF-1	21.41	26.56	36.24
HUVEC	28.89	28.2	29.57
MC116	14.07	37.97	49.64

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antibodies directed against the E2 envelope glycoprotein of hepatitis C virus. *Journal of virology*. 2009;83(23):12473-82.

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What is claimed is:

1. A single Kaposi sarcoma-associated herpesvirus-like particle (KSHV-LP) comprising two or more KSHV glycoproteins or an immunogenic fragment thereof.
2. The KSHV-LP of claim 1, wherein the two or more glycoproteins include gpK8.1, gB, and gH/gL.
3. The KSHV-LP of claim 1 or claim 2, further comprising one or more T cell antigens.
4. The KSHV-LP of claim 3, wherein the T cell antigen includes LANA1 or an immunogenic fragment thereof.
5. The KSHV-LP of any one of claims 1-4, further comprising one or more of NDV structural proteins.
6. The KSHV-LP of claim 5, wherein the one or more NDV structural proteins include fusion (F), matrix (M), nucleocapsid (NP).
7. A vaccine composition or a pharmaceutical composition comprising a therapeutically effective amount of the KSHV-LP of any one of claims 1-6.
8. A vaccine composition or a pharmaceutical composition comprising a therapeutically effective amount of a single KSHV-LP comprising two or more KSHV glycoproteins and one or more T cell antigens.
9. The vaccine composition or pharmaceutical composition of claim 8, wherein the two or more KSHV glycoproteins include gpK8.1, gB, and gH/gL.
10. The vaccine composition or pharmaceutical composition of claim 8 or claim 9, wherein the one or more T cell antigens include LANA1 or a fragment thereof.
11. The vaccine composition or pharmaceutical composition of any one of claims 8-10, wherein the single KSHV-LP comprises gpK8.1, gB, gH, gL, and LANA1 or a fragment thereof.

12. The vaccine composition or pharmaceutical composition of any one of claims 8-11, wherein the single KSHV-LP further comprises one or more of NDV structural proteins.
13. The vaccine composition or pharmaceutical composition of claim 12, wherein the one or more NDV structural proteins include fusion (F), matrix (M), nucleocapsid (NP).
14. The vaccine composition or the pharmaceutical composition of any one of claims 7-13, further comprising one or more adjuvants.
15. The vaccine composition or the pharmaceutical composition of any one of claims 7-14, further comprising one or more pharmaceutically acceptable carriers.
16. A method of preventing or treating a KSHV infection or a condition associated with a KSHV infection comprising administering to a subject in need thereof a therapeutically effective amount of the KSHV-LP of any one of claims 1-6.
17. A method of preventing or treating a KSHV infection or a condition associated with a KSHV infection comprising administering to a subject in need thereof the vaccine composition or pharmaceutical composition of any one of claims 7-15.
18. An immunization regimen comprising administering to a subject in need thereof one or more doses of a therapeutically effective amount of the KSHV-LP of any one of claims 1-6.
19. An immunization regimen comprising administering to a subject in need thereof one or more doses of the vaccine composition or pharmaceutical composition of any one of claims 7-15.
20. An expression system for co-expressing two or more KSHV envelope glycoproteins including a single vector inserted with two or more nucleic acid sequences that encode two or more KSHV envelope glycoproteins, linked by one or more linking sequences, such that the two or more KSHV envelope glycoproteins can be co-expressed simultaneously, self-cleaved and/or self-processed to assemble into one or more glycoprotein complexes.

21. The expression system of claim 20, wherein the one or more linking sequences include one or more 2A sequences encoding 2A peptides that mediate ribosomal skipping.

22. The expression system of claim 20 or claim 21, wherein the single vector is inserted with a single promoter before the two or more nucleic acid sequences such that the single promoter controls the expression of the two or more nucleic acid sequences.

23. A method of preventing or neutralizing KSHV infection in vitro comprising contacting a cell line permissive to KSHV infection with the KSHV-LP of any one of claims 1-6 such that a neutralizing antibody is produced.

24. The method of claim 23, wherein the cell line permissive to KSHV infection is a human cell line.

25. The method of claim 23 or claim 24, wherein the cell permissive to KSHV infection includes an epithelial cell line, an endothelial cell line, a fibroblast cell line, and a B cell line.

Fig. 1A

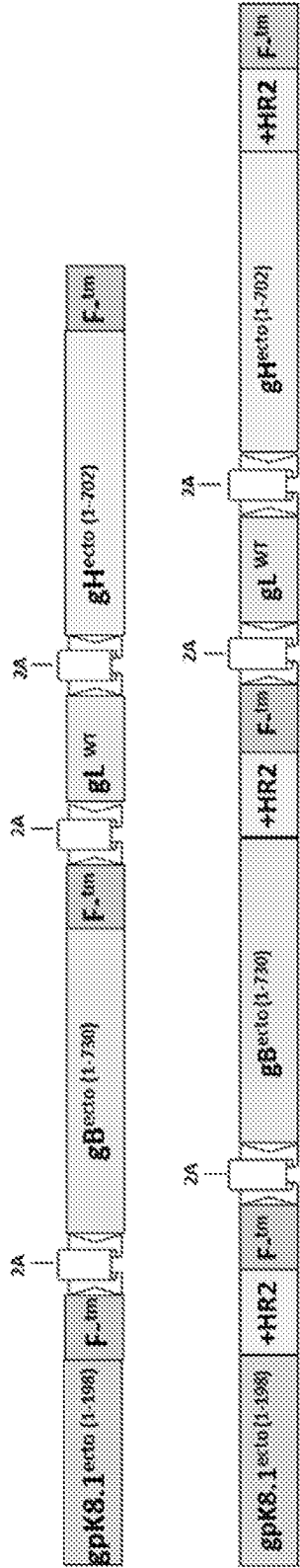


Fig. 1B

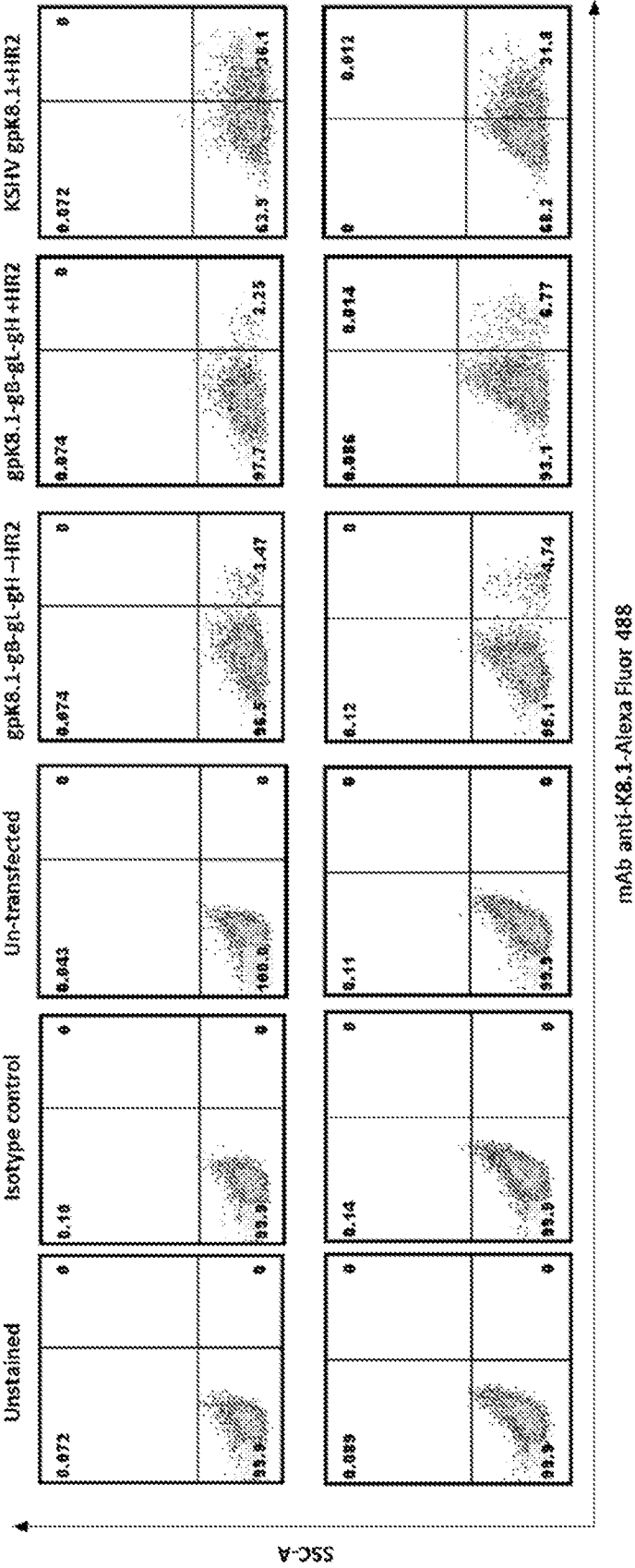


Fig. 2A

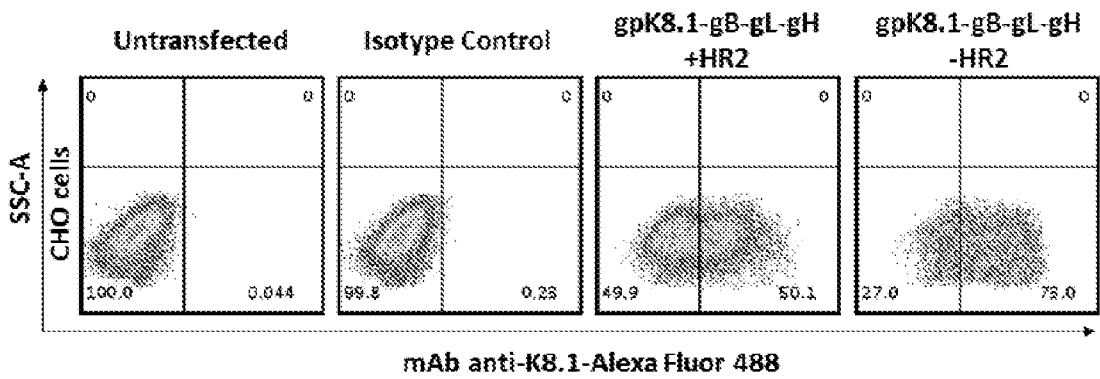


Fig. 2B

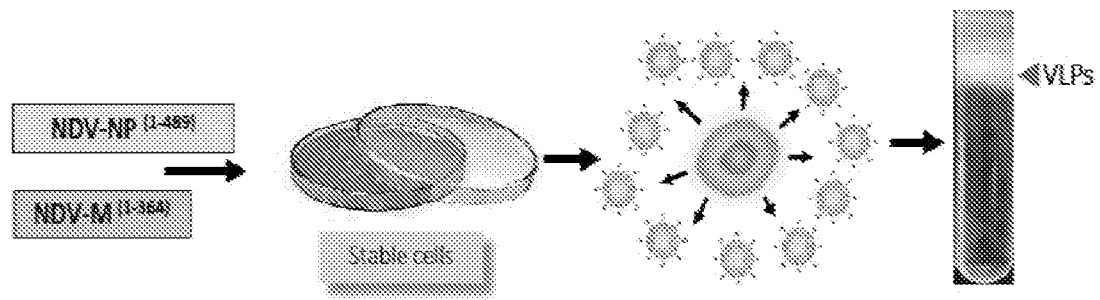


Fig. 2C

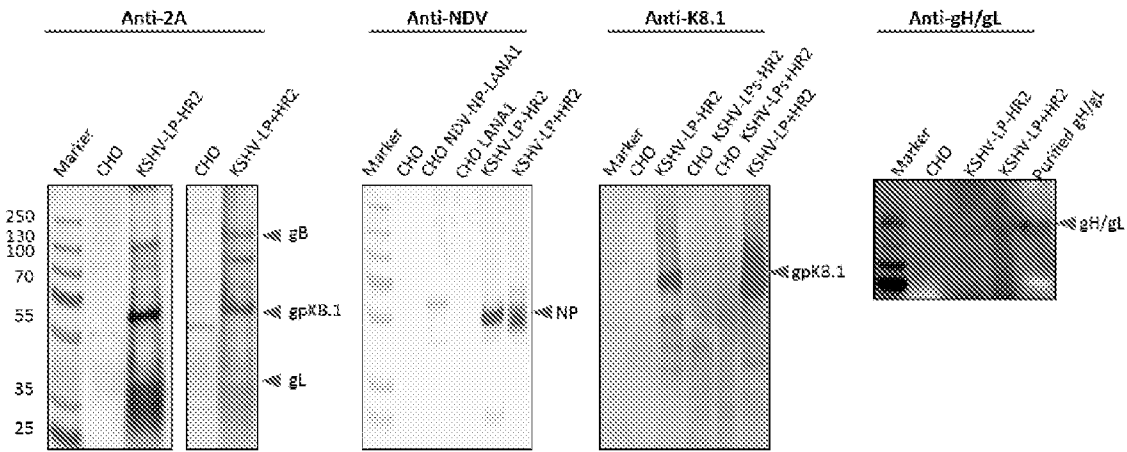


Fig. 2D

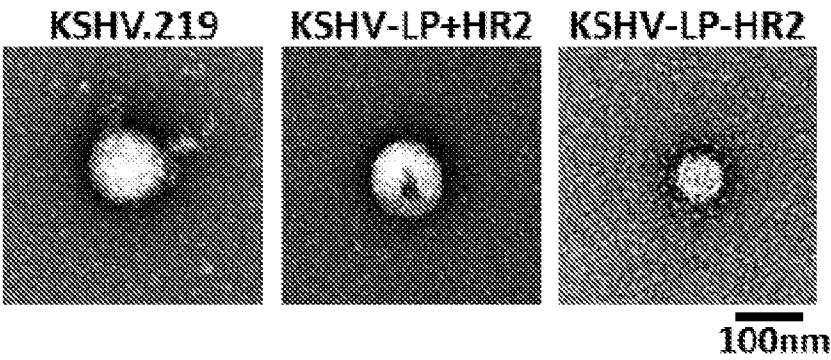


Fig. 3A

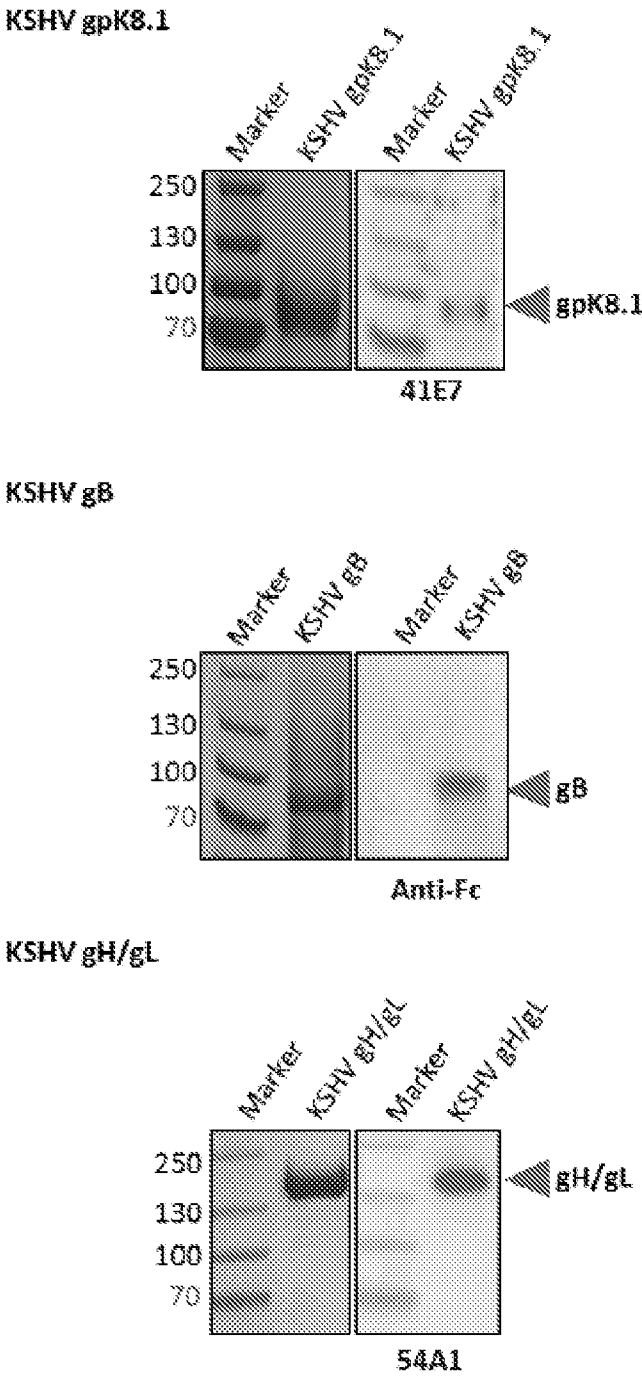


Fig. 3B

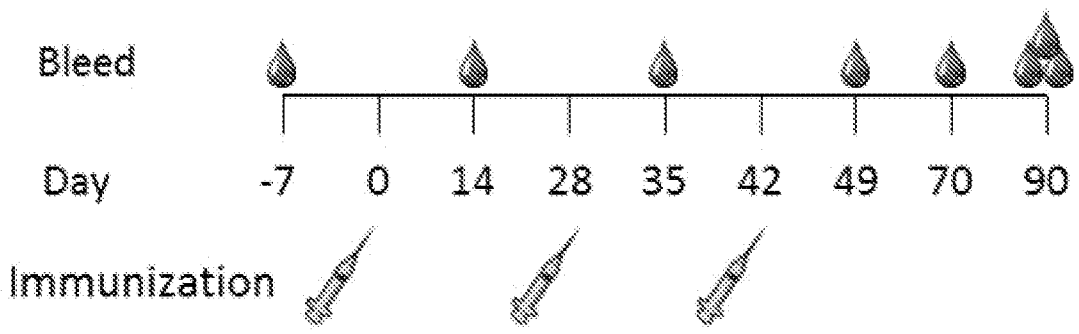


Fig. 3C

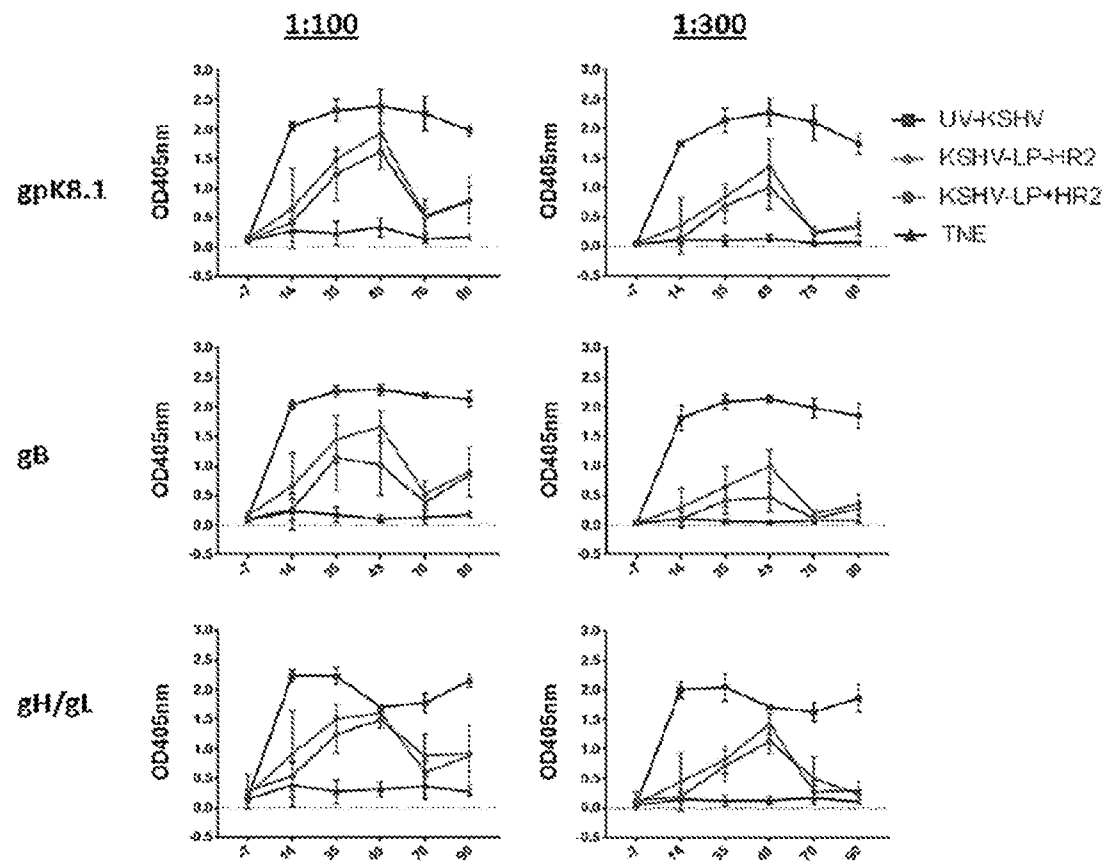


Fig. 4A

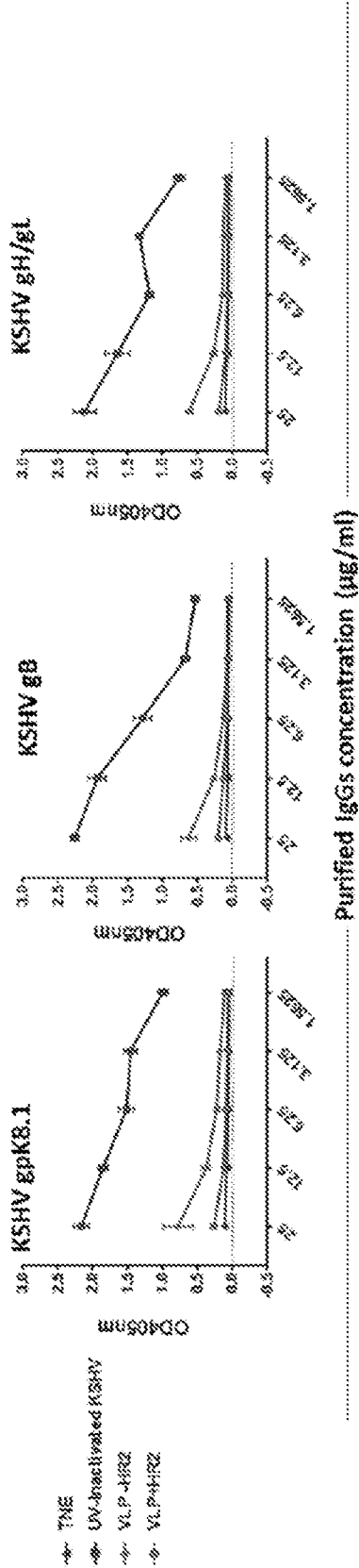


Fig. 4B

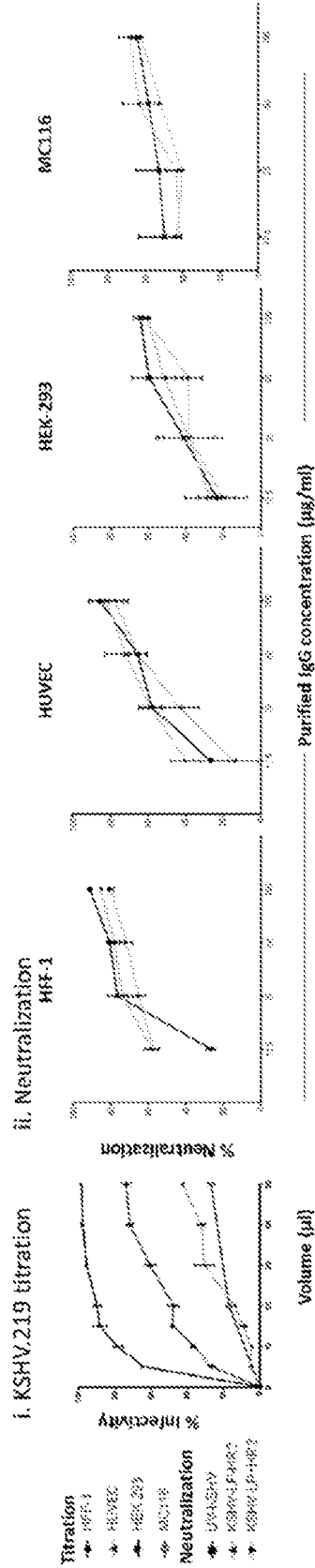
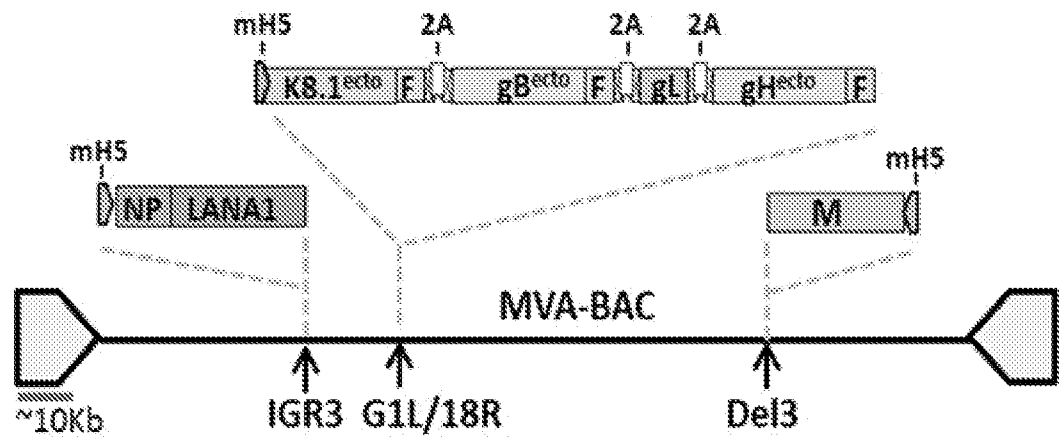


Fig. 5



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US20/19207

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61K 39/245; A61P 31/22; C07K 14/03; C12N 15/38 (2020.01)

CPC - A61K 39/245, 39/12; C07K 14/005; C12N 7/00

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	~ BARASA et al. 'BALB/c Mice Immunized With A Combination Of Virus-Like Particles Incorporating Kaposi Sarcoma-Associated Herpesvirus (KSHV) Envelope Glycoproteins GpK8.1, Gb, And Gh/Gl Induced Comparable Serum Neutralizing Antibody Activity To UV-inactivated KSHV' <i>Oncotarget</i> , 2017, Vol. 8, (No. 21), pp: 34481-34497; abstract; page 34482, column 2, second – fifth paragraphs; page 34491, column 1, first – second paragraph; page 34492, column 1, first paragraph	1-2, 3/1/2, 4/3/1-2, 8-9, 10/8-9 --- 20-21, 22/20-21
Y	WO 2007/014162 A2 (ABBOTT LABORATORIES) 01 February 2007; abstract; paragraphs [00013], [00022], [00026], [000185], [000295]	20-21, 22/20-21
A	US 9,402,895 B2 (CHANG et al.) 02 August 2016; entire document	1-2, 3/1/2, 4/3/1-2, 8-9, 10/8-9, 20-21, 22/20-21
P, X	~ MULAMA et al. 'A Multivalent Kaposi Sarcoma-Associated Herpesvirus-Like Particle Vaccine Capable Of Eliciting High Titers Of Neutralizing Antibodies In Immunized Rabbits' <i>Vaccine</i> . 2019 July 09; 37(30): 4184–4194; entire document. DOI:10.1016/j.vaccine.2019.04.071.	1-2, 3/1/2, 4/3/1-2, 8-9, 10/8-9, 20-21, 22/20-21

☐ 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

"D" document cited by the applicant in the international application

"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

20 April 2020 (20.04.2020)

Date of mailing of the international search report

20 JUL 2020

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Shane Thomas

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US20/19207

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.: 5-7, 11-19, 23-25
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.