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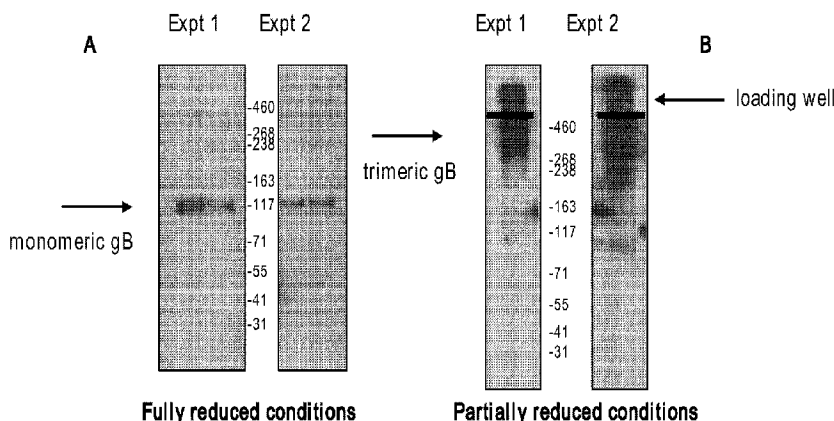
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(54) Titre : GLYCOPROTEINE B TRIMERE D'HERPESVIRUS HUMAIN, COMPLEXES PROTEIQUES COMPRENANT gB TRIMERE ET LEUR UTILISATION COMME VACCINS

(54) Title: HUMAN HERPESVIRUS TRIMERIC GLYCOPROTEIN B, PROTEIN COMPLEXES COMPRISING TRIMERIC gB AND THEIR USE AS VACCINES



(57) **Abrégé/Abstract:**

The present disclosure provides modified human herpesvirus glycoprotein B (gB) proteins that incorporate unique mechanisms for generating proteins that mimic their native conformation to enhance their immunogenicity. The modified herpesvirus gB proteins insert a peptide linker at the furin cleavage site in the extracellular domain of herpesvirus gB. When expressed, the gB subunit associates in triplicate to produce a homotrimeric complex, mimicking the native conformation of the gB protein. Also provided are protein complexes comprising a homotrimeric complex of a modified herpesvirus gB protein and herpesvirus gH and gL proteins. Also provided are nucleic acids encoding the modified herpesvirus gB proteins, methods of inducing or suppressing an immune response in a subject by administering a vaccine comprising the modified herpesvirus gB protein, or nucleic acid encoding the same, or a protein complex comprising a homotrimeric complex of the modified herpesvirus gB protein and herpesvirus gH and gL proteins.

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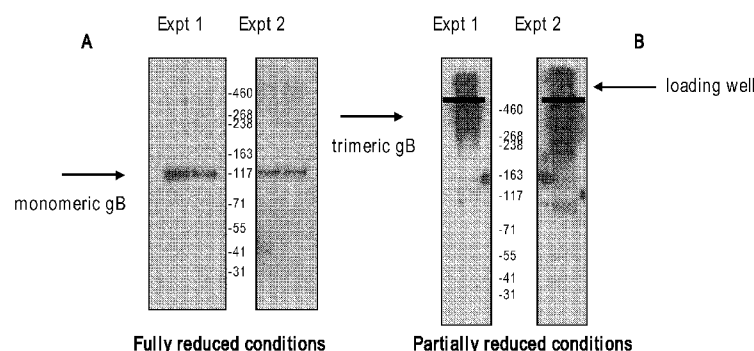


FIG. 1

(57) Abstract: The present disclosure provides modified human herpesvirus glycoprotein B (gB) proteins that incorporate unique mechanisms for generating proteins that mimic their native conformation to enhance their immunogenicity. The modified herpesvirus gB proteins insert a peptide linker at the furin cleavage site in the extracellular domain of herpesvirus gB. When expressed, the gB subunit associates in triplicate to produce a homotrimeric complex, mimicking the native conformation of the gB protein. Also provided are protein complexes comprising a homotrimeric complex of a modified herpesvirus gB protein and herpesvirus gH and gL proteins. Also provided are nucleic acids encoding the modified herpesvirus gB proteins, methods of inducing or suppressing an immune response in a subject by administering a vaccine comprising the modified herpesvirus gB protein, or nucleic acid encoding the same, or a protein complex comprising a homotrimeric complex of the modified herpesvirus gB protein and herpesvirus gH and gL proteins.

HUMAN HERPESVIRUS TRIMERIC GLYCOPROTEIN B, PROTEIN COMPLEXES COMPRISING TRIMERIC gB AND THEIR USE AS VACCINES

CROSS REFERENCE TO RELATED APPLICATIONS

[001] This application claims priority to U.S. provisional patent application number 61/914,903, filed 11 December 2013.

SEQUENCE LISTING

[002] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format. Said ASCII copy, created on December 11, 2014, is named HMJ-143-PCT_SL.txt and is 103,345 bytes in size.

BACKGROUND

[003] Human cytomegalovirus (HCMV) is a ubiquitously occurring pathogen that causes severe disease in immunocompromised hosts. HCMV is the most common viral infection acquired *in utero* in the developed world, and is a major cause of congenital defects in newborns. In the U.S. and Europe an estimated 0.2% to 1.2% of all live born infants are infected with HCMV. Congenital HCMV infection is a leading cause of sensorineural hearing loss in children and is the leading infectious cause of central nervous system damage in children.

[004] In addition to newborn infants, the virus can also cause severe disease in immunosuppressed patients, such as organ transplant recipients and HIV-positive individuals. HCMV can become an opportunistic pathogen in these patients and cause severe disease with high morbidity and mortality.

[005] HCMV is an enveloped, double-stranded DNA β -herpesvirus of the Herpesviridae family. Glycoprotein B (gB) of the Herpesviridae family is a type III fusion protein that has a shared trimeric structure of its fusion-active forms, and a post-fusion trimer of hairpins. HCMV gB is encoded by the UL55 gene and is synthesized as a 906-amino acid precursor molecule in infected cells. An amino-terminal signal sequence directs the nascent polypeptide to the endoplasmic reticulum (ER), where gB folds and rapidly associates into disulfide-dependent macromolecular complexes formed by identical molecules. Following transport from the ER, HCMV gB enters the Golgi apparatus, where it undergoes glycosylation and is processed by proteolysis by the host subtilisin-like enzyme, furin, into the amino-terminal and carboxy-terminal fragments, gp115 and gp55, respectively. These

two fragments of the monomeric form of gB – gp 115 and gp55 – are held together by intramolecular disulfide bonds. The mature product is then delivered to the surface of infected cells, where it is recycled between endosomal vesicles and the plasma membrane and is eventually incorporated into virions. Recently, native HCMV gB has been postulated to be a homotrimer based on the 3D crystallography structure of gB proteins in related viruses, Herpes Simplex Virus 1 (HSV-1) gB and Epstein Barr Virus (EBV) gB, which are homotrimers (29-32). Various vaccines, including live attenuated vaccines and subunit vaccines, are being developed to target HCMV-associated diseases. For example, gB is considered a major vaccine target antigen for eliciting neutralizing antibodies based on its critical role in mediating viral-host cell fusion and thus viral entry. Indeed a significant portion of neutralizing antibodies in human serum is specific for gB epitopes. Others have attempted to take advantage of this humoral response to gB in the effort to develop an effective vaccine for the prevention of HCMV infection. For example, a recombinant gB protein is described in Spaete et al., *A recombinant subunit vaccine approach to HCMV vaccine development*, Transplantation Proceedings, Vol 23, No 3, Suppl 3 (June), 1991: pp 90-96, and in WO 2012/049317. Based on analysis of a gB protein made in an analogous manner, it is believed that this recombinant gB protein is composed of mostly dimeric gB and minor amounts of monomeric and trimeric gB. This recombinant gB protein was generated by mutating the gene encoding for gB at the furin cleavage site, rendering the site ineffectual, and deleting the transmembrane domain, thus leaving the extracellular and intracellular domains.

[006] A vaccine based on this recombinant gB protein was used in Phase 2 clinical trials. Pass et al., *Vaccine Prevention of Maternal Cytomegalovirus Infection*, N Engl J Med 2009; 360:1191-1199. Three doses of the HCMV vaccine or placebo were given at 0, 1, and 6 months to HCMV-seronegative women within 1 year after they had given birth. HCMV infection was determined in the women in quarterly tests during a 42-month period, using an assay for IgG antibodies against HCMV proteins other than glycoprotein B. Infection was confirmed by virus culture or immunoblotting. The primary end point was the time until the detection of HCMV infection. 234 subjects were randomly assigned to receive the HCMV vaccine and 230 subjects to receive placebo. After a minimum of 1 year of follow-up, there were 49 confirmed infections, 18 in the vaccine group and 31 in the placebo group. Kaplan-Meier analysis showed that the vaccine group was more likely to remain uninfected during a 42-month period than the placebo group (P=0.02). Vaccine efficacy was 50% (95% confidence interval, 7 to 73) on the basis of infection rates per 100 person-years. One

congenital infection among infants of the subjects occurred in the vaccine group, and three infections occurred in the placebo group.

[007] However, no vaccine candidates for the prevention of HCMV have entered into Phase III clinical trials. Although the natural conformation of gB during HCMV infections is predicted to be a trimer, there has been no reported success in producing a recombinant trimeric gB.

[008] Likewise, Epstein-Barr Virus (EBV), also known as human herpesvirus 4, is a major, global source of morbidity and mortality, responsible for such pathologic entities as Burkitt lymphoma, nasopharyngeal carcinoma, infectious mononucleosis, a subset of Hodgkin's disease, and the lymphoproliferative syndrome in immunosuppressed patients. EBV is a γ -herpesvirus, with a double stranded, linear DNA genome, that infects B cells and epithelial cells. Vaccines being developed to target EBV infection have focused on glycoprotein 350 (gp350) (E.M. Sokal et al., *J. Infect. Dis.* 196: 1749 (2007)); however, no vaccine candidates for the prevention of EBV have targeted EBV gB, whose natural conformation during EBV infection has been shown to be a trimer. (Backovic M, Longnecker R, Jardetzky TS. 2009. Structure of a trimeric variant of the Epstein-Barr virus glycoprotein B. *Proc Natl Acad Sci U S A* 106: 2880-5.)

SUMMARY

[009] The present disclosure provides new and improved strategies for enhancing an immune response to herpesvirus infection. These improved strategies involve creating a modified herpesvirus gB by inserting a peptide linker at the furin cleavage site in the herpesvirus gB polypeptide extracellular domain. Inserting the peptide linker removes the furin recognition sequence, such that expression of the modified herpesvirus gB results in the production of a homotrimeric gB complex that provides enhanced immunogenicity. Without intending to be bound by any theory, it is believed that such a linker sequence can allow the modified herpesvirus gB polypeptide to undergo native conformational folding and form a homotrimer.

[0010] In one embodiment there is provided a human herpesvirus glycoprotein B (gB) polypeptide comprising a modified extracellular domain or fragment thereof, wherein the modified extracellular domain or fragment thereof comprises a peptide linker inserted into the furin cleavage site, wherein the peptide linker is 6 to about 70 amino acids in length and wherein expression of the modified human herpesvirus gB polypeptide results in the formation of a homotrimeric complex.

[0011] Another aspect is a recombinant nucleic acid encoding the modified herpesvirus gB polypeptide, and a method of using the recombinant nucleic acid to express the modified herpesvirus gB polypeptide in a cell. Yet another aspect is directed to a method of inducing an immune response in a subject by administering to the subject a vaccine composition comprising the modified herpesvirus gB polypeptide or a nucleic acid encoding the same, where the herpesvirus gB polypeptide induces an immune response in the subject. The vaccine composition can optionally include other herpesvirus antigens, including but not limited to one or more of glycoprotein H (gH), glycoprotein L (gL), glycoprotein 350 (gp350), UL128, UL130, UL131, or combinations thereof.

[0012] Another aspect is directed to a protein complex comprising a herpesvirus gB polypeptide homotrimer complex, a herpesvirus gH glycoprotein, and a herpesvirus gL glycoprotein, where the herpesvirus gB polypeptide homotrimer complex comprises a trimer of three modified herpesvirus gB polypeptides. In certain embodiments, the herpesvirus gH and gL glycoproteins comprises a herpesvirus gH/gL fusion protein. In certain embodiments, the protein complex further comprises one or more of a herpesvirus UL128, UL130, or UL131 polypeptide.

[0013] In another embodiment there is provided a complex comprising a herpesvirus gB polypeptide homotrimer complex, a herpesvirus glycoprotein H (gH), and a glycoprotein L (gL), wherein the homotrimer complex comprises three human herpesvirus gB polypeptides and each human herpesvirus glycoprotein B (gB) polypeptide comprises a modified extracellular domain or fragment thereof, wherein the modified extracellular domain or fragment thereof comprises a peptide linker inserted into the furin cleavage site, wherein the peptide linker is from 6 to about 70 amino acids in length.

[0014] Also provided are methods of making the protein complex, comprising incubating *in vitro* a first protein and at least a second protein to form the protein complex. In one embodiment, the method of making the protein complex comprises incubating *in vitro* a herpesvirus gB polypeptide homotrimer complex, a herpesvirus gH glycoprotein, and a herpesvirus gL glycoprotein, where the herpesvirus gB polypeptide homotrimer complex comprises a trimer of three modified herpesvirus gB polypeptides, and forming the protein complex. In certain embodiments, the herpesvirus gH and gL glycoproteins comprises a herpesvirus gH/gL fusion protein. In certain embodiments, the method further comprises incubating one or more of a herpesvirus UL128, UL130, or UL131 polypeptide. Thus, in certain embodiments, the method comprises incubating a homotrimeric complex of a

modified herpesvirus gB protein, a herpesvirus gH/gL fusion protein, and optionally a herpesvirus UL128, a herpesvirus UL130, and a herpesvirus UL131 polypeptide.

[0015] Also provided are methods of inducing an immune response in a subject by administering to the subject a vaccine composition comprising the herpesvirus protein complex, where the herpesvirus protein complex induces an immune response in the subject. The herpesvirus protein complex comprises a herpesvirus gB polypeptide homotrimer complex, a herpesvirus gH glycoprotein, and a herpesvirus gL glycoprotein, where the herpesvirus gB polypeptide homotrimer complex comprises a trimer of three modified herpesvirus gB polypeptides. In certain embodiments, the herpesvirus gH and gL glycoproteins comprises a herpesvirus gH/gL fusion protein. In certain embodiments, the protein complex further comprises one or more of a herpesvirus UL128, UL130, or UL131 polypeptide. Thus, in certain embodiments, the protein complex comprises a homotrimeric complex of a modified herpesvirus gB protein, a herpesvirus gH/gL fusion protein and optionally a herpesvirus UL128, a herpesvirus UL130, and a herpesvirus UL131A polypeptide.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate certain embodiments, and together with the written description, serve to explain certain principles of the constructs and methods disclosed herein.

[0017] **Figure 1A** shows a Western blot analysis of HCMV gB under fully reduced conditions using an anti-His monoclonal antibody.

[0018] **Figure 1B** shows a Western blot analysis of HCMV gB under partially reduced conditions using an anti-His monoclonal antibody.

[0019] **Figure 2A** shows a Western blot under modified native conditions using an anti-HCMV gB monoclonal antibody.

[0020] **Figure 2B** shows a Western blot under reduced conditions using an anti-HCMV gB monoclonal antibody.

[0021] **Figure 3** depicts a schematic difference between a wild type HCMV gB and a modified HCMV gB of the present disclosure.

[0022] **Figure 4** shows that a modified HCMV gB of this disclosure (“Trimer”) is markedly more immunogenic than non-trimeric control HCMV gB (“Sino”), which is nearly identical to what was used in the Phase II clinical trial by Pass et al., *Vaccine Prevention of*

Maternal Cytomegalovirus Infection, N Engl J Med 2009; 360:1191-1199. Significance between modified HCMV gB and control protein, $p < 0.05$ by Student-t test.

[0023] **Figure 5** shows serum titers of HCMV gB-specific IgG in rabbits immunized with trimeric HCMV gB on days 0, 21, and 42.

[0024] **Figures 6A-B** show the *in vitro* neutralizing activity of serum from rabbits immunized with trimeric HCMV gB against live HCMV using MRC-5 fibroblasts (**A**) and ARPE-19 epithelial cells (**B**). Human serum from a CMV-immune patient was used as a positive control (“Human sera”).

[0025] **Figure 7A** shows a Western blot analysis of trimeric EBV gB under reducing conditions using an anti-His monoclonal antibody.

[0026] **Figures 7B-C** show a Western blot analysis of trimeric EBV gB under non-reducing conditions using an anti-His monoclonal antibody (**B**) or an anti-EBV gB antibody (**C**).

[0027] **Figure 8** depicts a schematic difference between a wild type EBV gB and a modified EBV gB of the present disclosure.

[0028] **Figures 9A-B** show a Western blot analysis of HCMV gH/gL heterodimer under reducing conditions using an anti-His monoclonal antibody (**A**) or an anti-HCMV gH antibody (**B**).

[0029] **Figure 10** shows a Western blot analysis of HCMV gB/gH/gL protein complex under non-reducing conditions.

DETAILED DESCRIPTION

[0030] It is to be understood that the following detailed description is provided to give the reader a fuller understanding of certain embodiments, features, and details of aspects of the invention, and should not be interpreted as a limitation of the scope of the invention.

[0031] **Definitions.** In order that the present invention may be more readily understood, certain terms are first defined. Additional definitions are set forth throughout the detailed description.

[0032] The term “**peptide linker**” refers to a short, non-native peptide sequence that links two proteins or fragments of a protein.

[0033] The term “**herpesvirus gH/gL fusion protein**” refers to a recombinant fusion protein comprising a herpesvirus gH protein joined to a herpesvirus gL protein. The herpesvirus gH protein can be joined to the herpesvirus gL protein with a peptide linker.

[0034] The term “**modified extracellular domain**” refers to the extracellular domain of a human herpesvirus glycoprotein B that has been engineered such that the amino acid sequence is not the native amino acid sequence. As used herein, the extracellular domain of the human herpesvirus glycoprotein B has been modified by inserting a peptide linker at the furin cleavage site, effectively removing the furin recognition sequence. Examples of such human herpesviruses include, but are not limited to, CMV (Cytomegalovirus), HSV-1 (Herpes Simplex Virus-1), HSV-2 (Herpes Simplex Virus-2), VZV (Varicella-Zoster Virus), EBV (Epstein-Barr Virus), and HSHV (Kaposi Sarcoma-related Herpes Virus).

[0035] The terms “**modified herpesvirus gB**” and “**modified herpesvirus gB polypeptide**” are used interchangeably and refer to a human herpesvirus glycoprotein B polypeptide comprising a modified extracellular domain.

[0036] The terms “**modified HCMV gB**” and “**modified HCMV gB polypeptide**” are used interchangeably and refer to a human CMV glycoprotein B polypeptide comprising a modified extracellular domain.

[0037] The terms “**modified EBV gB**” and “**modified EBV gB polypeptide**” are used interchangeably and refer to a human Epstein Barr virus glycoprotein B polypeptide comprising a modified extracellular domain.

[0038] The term “**leader sequence**” refers to a short peptide sequence at the N-terminus of a recombinant protein that directs the recombinant protein to be secreted by the cell.

[0039] The terms “**homotrimer**,” “**homotrimer complex**,” and “**homotrimeric complex**” are used interchangeably and refer to the association of three polypeptides, such as three modified herpesvirus or HCMV gB polypeptides.

[0040] The term “**pharmaceutically acceptable carrier**” or “**pharmaceutically acceptable excipient**” means solvents, dispersion media, coatings, antibacterial agents and antifungal agents, isotonic agents, and absorption delaying agents, and the like, that are compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. In certain embodiments, the pharmaceutically acceptable carrier or excipient is not naturally occurring.

[0041] The term “**isolated**,” when used in the context of a polypeptide or nucleic acid refers to a polypeptide or nucleic acid that is substantially free of its natural environment and is thus distinguishable from a polypeptide or nucleic acid that might happen to occur naturally. For instance, an isolated polypeptide or nucleic acid is substantially free of cellular material or other polypeptides or nucleic acids from the cell or tissue source from which it

was derived. The term also refers to preparations where the isolated polypeptide or nucleic acid is sufficiently pure for pharmaceutical compositions; or at least 70-80% (w/w) pure; or at least 80-90% (w/w) pure; or at least 90-95.

[0042] The terms “**polypeptide**,” “**peptide**,” and “**protein**” are used interchangeably herein to refer to polymers of amino acids.

[0043] The term “**recombinant**” when used in the context of a nucleic acid means a nucleic acid having nucleotide sequences that are not naturally joined together and can be made by artificially combining two otherwise separated segments of sequence. This artificial combination is often accomplished by chemical synthesis or, more commonly, by the artificial manipulation of isolated segments of nucleic acids, for example, by genetic engineering techniques. Recombinant nucleic acids include nucleic acid vectors comprising an amplified or assembled nucleic acid, which can be used to transform or transfect a suitable host cell. A host cell that comprises the recombinant nucleic acid is referred to as a “recombinant host cell.” The gene is then expressed in the recombinant host cell to produce a “recombinant polypeptide.” A recombinant nucleic acid can also serve a non-coding function (for example, promoter, origin of replication, ribosome-binding site and the like).

[0044] A homotrimeric herpesvirus gB, in contrast to the previously tested non-trimeric gB by Pass et al., *Vaccine Prevention of Maternal Cytomegalovirus Infection*, N Engl J Med 2009; 360:1191-1199, is likely to elicit higher total gB-specific IgG responses and more diverse neutralizing antibodies against herpesvirus due to its multimeric form and the likely expression of unique conformational, neutralizing epitopes by homotrimeric herpesvirus gB. Thus, new and improved constructs for enhancing immune responses are needed, particularly herpesvirus gB constructs, including but not limited to HCMV gB constructs, that can be used to enhance immune responses in response to herpesvirus infection.

[0045] **Modified Herpesvirus/HCMV gB.** The nucleic acid sequence encoding for wild type HCMV gB is set forth in SEQ ID NO: 1. The polypeptide sequence of wild type HCMV gB is set forth in SEQ ID NO: 2. Wild type HCMV gB is expressed as a 906 amino acid precursor protein. The first 22 amino acids comprise the native signal peptide, which sends the precursor protein to the endoplasmic reticulum (ER) for processing. The native signal peptide is cleaved off when the protein is folded in the ER. The polypeptide sequence of wild type HCMV gB consists of an extracellular domain (amino acids 23-750 of SEQ ID NO: 2), a transmembrane domain, and an intracellular domain (together, amino acids 751-906 of SEQ ID NO: 2).

[0046] Following transport from the ER, HCMV gB enters the Golgi apparatus where it undergoes glycosylation and is cleaved by the host enzyme, furin, in the extracellular domain at amino acids 460-461 of SEQ ID NO: 2. This proteolytic processing gives rise to two polypeptide fragments, gp116 and gp55. These two fragments remain covalently associated by disulfide bonds to form a gB subunit. It is believed that three HCMV gB subunits associate to create a homotrimer complex that mediates viral-host cell fusion (29-32).

[0047] The present disclosure relates to a new strategy for generating a modified herpesvirus gB polypeptide. The present disclosure describes a strategy for generating a modified HCMV gB polypeptide; however, it is understood that the strategy is not limited to HCMV and can be broadly applied across human herpesviruses, which share a homologous gB structure, including a furin cleavage site in the extracellular domain. Examples of such human herpesviruses include, but are not limited to, CMV, HSV-1 (Herpes Simplex Virus-1), HSV-2 (Herpes Simplex Virus-2), VZV (Varicella-Zoster Virus), EBV (Epstein-Barr Virus), and HSHV (Kaposi Sarcoma-related Herpes Virus). The nucleotide and amino acid sequences of the gB polypeptides of CMV, HSV-1, HSV-2, VZV, EBV, and HSHV are known.

[0048] The strategy involves creating nucleic acid constructs for inserting a peptide linker at the furin cleavage site in the extracellular domain of a herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, and HSHV) gB such that the encoded herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, and HSHV) gB forms a subunit that associates in triplicate to produce a gB trimeric complex. Surprisingly, modified HCMV gB polypeptide produced according to the present disclosure uniformly and consistently forms a homotrimeric complex. Without being limited by theory, it is believed that mutating the furin cleavage site in HCMV gB so that said site is rendered ineffectual, as had been done previously (*see* Spaete et al.), limits the movement of the HCMV gp116 and gp55 fragments, thereby interfering with the fragments' ability to form a homotrimeric complex. This could account for the inability of the previously described recombinant HCMV gB proteins to fold into a homotrimer. Replacing the furin cleavage site with a peptide linker, on the other hand, allows the gB polypeptide to form a trimeric complex, similar to the homotrimer that is believed to form naturally in a cell.

[0049] In certain embodiments, the modified herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, and HSHV) gB polypeptide of the present disclosure comprises a modified extracellular domain of wild type herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, and

HSHV) gB and does not include the transmembrane domain or the intracellular domain of wild type herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, and HSHV) gB. The modified herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, and HSHV) gB generally retains one or more characteristics of the corresponding native herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, and HSHV) gB, such as the ability to mediate viral-host cell fusion, or the ability to elicit antibodies (including, but not limited to, viral neutralizing antibodies) capable of recognizing native herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, and HSHV) gB. Conventional methodology may be utilized to evaluate modified herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, and HSHV) gB for one or more of the characteristics.

[0050] By way of example, and not limitation, the polynucleotide sequence can include nucleotides encoding for a leader sequence that is not the native herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, and HSHV) gB leader sequence (e.g., the leader sequence is not amino acids 1-22 of SEQ ID NO: 2 for a modified HCMV gB polypeptide). In other embodiments, the polynucleotide sequence includes nucleotides encoding a protein comprising the amino acid sequence of SEQ ID NO: 4. In further embodiments, the polynucleotide sequence comprises SEQ ID NO: 3, which includes nucleotides encoding an IgG κ leader sequence. In an embodiment, the IgG κ leader sequence has the amino acid sequence METDTLLLWVLLLWVPGSTGD (SEQ ID NO: 6).

[0051] In an aspect, the modified HCMV gB was created with a nucleic acid construct encoding for the extracellular domain of wild type HCMV gB (amino acids 23-750 of SEQ ID NO: 2) but replacing the furin cleavage sequence (amino acids 457-461 of SEQ ID NO: 2) with a peptide linker, such as ((Gly₄Ser)₃ (SEQ ID NO: 5)). The nucleic acid sequence encoding for the modified HCMV gB is set forth in SEQ ID NO: 3. The polypeptide sequence of the modified HCMV gB is set forth in SEQ ID NO: 4. In one embodiment, the modified HCMV gB comprises only the extracellular domain which includes the gp116 and gp55 fragments joined together with the peptide linker. This modified HCMV gB construct uniformly forms a homotrimeric complex when expressed, as compared to the traditional non-trimeric HCMV gB protein produced by prior methods. This strategy for creating modified HCMV gB can be exploited with other peptide linkers in varying lengths and compositions as described below. This strategy for creating modified HCMV gB can result in a composition wherein the modified HCMV gB comprise at least 70%, for example at least 75%, 80%, 85%, or 90% homotrimers.

[0052] In another aspect, the modified HCMV gB can be created with the insertion of a peptide linker at the furin cleavage site between amino acid residues 460 and 461 without deleting any of the amino acid residues of the furin recognition sequence RTKRS (SEQ ID NO: 19). In yet another aspect, insertion of a peptide linker at the furin cleavage site can be coupled with deletion of 1, 2, 3, 4, or 5 amino acid residues of the furin recognition sequence RTKRS (SEQ ID NO: 19).

[0053] In a further aspect, the modified HCMV gB can comprise a partial sequence of the amino acid residues 23-460 of wild type HCMV gB at the amino terminal end of the peptide linker, and a partial sequence of the amino acid residues 461-750 of wild type HCMV gB at the carboxyl terminal end of the peptide linker.

[0054] This strategy for inserting a peptide linker at the cleavage site within a protein, with or without deleting part of or the entire enzyme recognition sequence, to achieve correct protein folding can be exploited with proteins other than herpesvirus glycoprotein B, including other viral, bacterial, parasitic, autoimmune, and tumor antigenic proteins. Thus, one aspect is directed to a recombinant polypeptide comprising a peptide linker that disrupts an enzymatic cleavage sequence, such as a furin cleavage sequence, that is present in the wild type form of the polypeptide. This platform can be used to create recombinant multimeric proteins that achieve correct native folding patterns without enzymatic cleavage when expressed in a host cell. For example, a homo- or heterodimer, homo- or heterotrimer, or tetramer can be created by inserting a peptide linker(s) at the cleavage site(s) responsible for multimeric formation. The encoded protein construct will form the appropriate naturally-occurring multimer without enzymatic cleavage by the host cell. In an aspect, a recombinant nucleic acid is contemplated that encodes the modified protein, and a method of using the recombinant nucleic acid to express the modified protein in a cell. In yet another aspect, it is contemplated methods of inducing an immune response in a subject by administering to the subject a vaccine composition comprising the modified protein or a nucleic acid encoding the same, where the modified protein induces an immune response in the subject.

[0055] ***Modified EBV gB***

[0056] The nucleic acid sequence encoding for wild type EBV gB is set forth in SEQ ID NO: 7. The polypeptide sequence of wild type EBV gB is set forth in SEQ ID NO: 8. As with the HCMV gB, proteolytic processing of gB gives rise to two segments, which remain covalently associated by disulfide bonds to form a gB subunit.

[0057] In an aspect, the modified EBV gB comprises a nucleic acid construct encoding for the extracellular domain of wild type EBV gB (amino acids 23-732 of SEQ ID NO: 8) but replacing the furin cleavage sequence (amino acids 429-433 of SEQ ID NO: 8) with a peptide linker, such as ((Gly₄Ser)₃ (SEQ ID NO: 5)). The nucleic acid sequence encoding for the modified EBV gB is set forth in SEQ ID NO: 9. The polypeptide sequence of the modified EBV gB is set forth in SEQ ID NO: 10. In one embodiment, the modified EBV gB comprises only the extracellular domain which includes the two fragments joined together with the peptide linker. This modified EBV gB construct would uniformly form a homotrimeric complex when expressed. This strategy for creating modified EBV gB can be exploited with other peptide linkers in varying lengths and compositions as described below. This strategy for creating modified EBV gB can result in a composition wherein the modified EBV gB comprise at least 70%, for example at least 75%, 80%, 85%, or 90% homotrimers.

[0058] In another aspect, the modified EBV gB can be created with the insertion of a peptide linker at the furin cleavage site between amino acid residues 432 and 433 without deleting any of the amino acid residues of the furin recognition sequence RRRRD (SEQ ID NO: 20). In yet another aspect, insertion of a peptide linker at the furin cleavage site can be coupled with deletion of 1, 2, 3, 4, or 5 amino acid residues of the furin recognition sequence RRRRD (SEQ ID NO: 20).

[0059] In a further aspect, the modified EBV gB can comprise a partial sequence of the amino acid residues 23-432 of wild type EBV gB at the amino terminal end of the peptide linker, and a partial sequence of the amino acid residues 433-732 of wild type EBV gB at the carboxyl terminal end of the peptide linker.

[0060] ***Peptide Linker Sequences.*** In the modified herpesvirus gB polypeptides (e.g., HCMV gB, HSV-1 gB, HSV-2 gB, VZV gB, EBV gB, or HSHV gB), linker sequences are inserted at the furin cleavage site. For example, the gp116 and gp55 fragments naturally formed when wild type HCMV gB is enzymatically cleaved by furin are joined by the peptide linker in the modified HCMV gB of the present invention. It is understood that the peptide linker is a non-native sequence that does not naturally exists in the native protein sequence.

[0061] In one embodiment, the linker sequence is a polypeptide having 5-70 amino acids, particularly a length of 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 30, 35, 40, 45, 50, 55, 60, 65, or 70 amino acids. In another embodiment, the linker sequence is a polypeptide having 10-25 amino acids. The linker sequence preferably

comprises glycine and serine amino acids. In one embodiment, the linker sequence is 15 amino acids in length and has the amino acid sequence (Gly₄Ser)₃ (SEQ ID NO:5).

[0062] Other suitable peptide linkers are those described in U.S. Pat. Nos. 4,751,180, 4,935,233, and 5,073,627. A DNA sequence encoding a desired linker sequence may be inserted in place of, and in the same reading frame as, for example, DNA sequences encoding one or more amino acids of the native furin cleavage site (e.g., RTKRS (SEQ ID NO: 19) in HCMV or RRRRD (SEQ ID NO: 20) in EBV) using conventional techniques known in the art. For example, a chemically synthesized oligonucleotide encoding the linker may be ligated in the full polynucleotide sequence to be inserted at the sequences encoding the native furin cleavage site.

[0063] **Protein Complexes.** The present disclosure also provides protein complexes comprising a herpesvirus gB polypeptide homotrimer complex, a herpesvirus gH glycoprotein, and a herpesvirus gL glycoprotein, where the herpesvirus gB polypeptide homotrimer complex comprises a trimer of three modified herpesvirus gB polypeptides. In certain embodiments, the herpesvirus gH and gL glycoproteins are part of a herpesvirus gH/gL fusion protein. In other embodiments, the protein complex further comprises one or more of a herpesvirus UL128, UL130, or UL131 polypeptide. Also provided are vaccine compositions comprising the protein complexes and a pharmaceutically acceptable carrier and/or an adjuvant.

[0064] Proteins in the protein complex are linked by non-covalent protein-protein interactions, including but not limited to hydrogen bonding and salt bridges. The protein complex has a quaternary structure, corresponding to the arrangement or shape resulting from the assembly and interaction of the individual proteins, and, therefore, is useful for inducing neutralizing antibodies against conformation epitopes on the gB/gH/gL protein complex. The protein complex, as used herein, does not refer to the native protein complex as it exists on the surface of a herpesvirus. Rather, the protein complex is formed by incubating the individual proteins *in vitro*, to create a reconstructed protein complex. There have been no reports demonstrating that these herpesvirus proteins, in their natural conformation, assemble into a native complex upon *in vitro* co-incubation.

[0065] The present disclosure describes a strategy for generating a herpesvirus gB/gH/gL protein complex as discussed above, which can be applied to any human herpesviruses including, but not limited to, CMV, HSV-1 (Herpes Simplex Virus-1), HSV-2 (Herpes Simplex Virus-2), VZV (Varicella-Zoster Virus), EBV (Epstein-Barr Virus), and HSHV (Kaposi Sarcoma-related Herpes Virus). The nucleotide and amino acid sequences of

the gB, gH, and gL polypeptides of CMV, HSV-1, HSV-2, VZV, EBV, and HSHV are known.

[0066] ***Nucleic Acids, Cloning and Expression Systems.*** The present disclosure further provides isolated nucleic acids encoding the disclosed modified herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, or HSHV) gB polypeptides. The nucleic acids may comprise DNA or RNA and may be wholly or partially synthetic or recombinant. Reference to a nucleotide sequence as set out herein encompasses a DNA molecule with the specified sequence, and encompasses a RNA molecule with the specified sequence in which U is substituted for T, unless context requires otherwise.

[0067] The present disclosure also provides constructs in the form of plasmids, vectors, phagemids, transcription or expression cassettes which comprise at least one nucleic acid encoding a modified herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, or HSHV) gB. The disclosure further provides a host cell which comprises one or more constructs as above.

[0068] Also provided are methods of making the modified herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, or HSHV) gB polypeptides encoded by these nucleic acids. The modified herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, or HSHV) gB proteins may be produced using recombinant techniques. The production and expression of recombinant proteins is well known in the art and can be carried out using conventional procedures, such as those disclosed in Sambrook et al., *Molecular Cloning: A Laboratory Manual* (4th Ed. 2012), Cold Spring Harbor Press. For example, expression of the modified herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, or HSHV) gB protein may be achieved by culturing under appropriate conditions recombinant host cells containing the nucleic acid encoding the herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, or HSHV) gB protein. Following production by expression the modified herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, or HSHV) gB protein may be isolated and/or purified using any suitable technique, then used as appropriate.

[0069] Systems for cloning and expression of a polypeptide in a variety of different host cells are well known in the art. Any protein expression system compatible with the constructs disclosed in this application may be used to produce the modified herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, or HSHV) gB protein.

[0070] Suitable vectors can be chosen or constructed, so that they contain appropriate regulatory sequences, including promoter sequences, terminator sequences, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate.

[0071] A further aspect of the disclosure provides a host cell comprising a nucleic acid as disclosed herein. A still further aspect provides a method comprising introducing such nucleic acid into a host cell. The introduction may employ any available technique. For eukaryotic cells, suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection and transduction using retrovirus or other virus, e.g., vaccinia or, for insect cells, baculovirus. For bacterial cells, suitable techniques may include calcium chloride transformation, electroporation and transfection using bacteriophage. These techniques are well known in the art. *See e.g.*, Current Protocols in Molecular Biology, Ausubel et al. eds., John Wiley & Sons (2010). DNA introduction may be followed by a selection method (e.g., antibiotic resistance) to select cells that contain the vector.

[0072] ***Vaccine Compositions.*** The modified herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, or HSHV) gB polypeptides and nucleic acids encoding the same and herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, or HSHV) gB/gH/gL protein complexes that are described in this application provide an improved platform for developing a vaccine that achieves enhanced immunogenicity in a subject. A homotrimeric complex of modified herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, or HSHV) gB or a protein complex comprising the homotrimeric complex of modified herpesvirus gB, in contrast to previously disclosed non-trimeric gB, is likely to elicit higher total gB-specific IgG responses and more diverse neutralizing antibodies against HCMV due to its multimeric form and the likely expression of unique conformational, neutralizing epitopes by trimeric gB. Thus, one embodiment is directed to a composition comprising the nucleic acid encoding the modified herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, or HSHV) gB protein and at least one pharmaceutically acceptable excipient. Another embodiment is directed to a composition comprising a homotrimeric complex of the modified herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, or HSHV) gB protein, at least one pharmaceutically acceptable excipient, and optionally an adjuvant. Yet another embodiment is directed to a composition comprising a protein complex, wherein the protein complex comprises a homotrimeric complex of the modified herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, or HSHV) gB protein, a herpesvirus gH/gL fusion protein, at least one pharmaceutically acceptable excipient, and optionally an adjuvant. These compositions are collectively referred to as “vaccine composition.” In certain embodiments, the vaccine composition does not include an adjuvant.

[0073] The pharmaceutically acceptable excipient can be chosen from, for example, diluents such as starch, microcrystalline cellulose, dicalcium phosphate, lactose, sorbitol, mannitol, sucrose, methyl dextrans; binders such as povidone, hydroxypropyl methylcellulose, dihydroxy propylcellulose, and sodium carboxymethylcellulose; and disintegrants such as crospovidone, sodium starch glycolate, croscarmellose sodium, and mixtures of any of the foregoing. The pharmaceutically acceptable excipient can further be chosen from lubricants such as magnesium stearate, calcium stearate, stearic acid, glyceryl behenate, hydrogenated vegetable oil, glycerine fumerate and glidants such as colloidal silicon dioxide, and mixtures thereof. In some embodiments, the pharmaceutically acceptable excipient is chosen from microcrystalline cellulose, starch, talc, povidone, crospovidone, magnesium stearate, colloidal silicon dioxide, sodium dodecyl sulfate, and mixtures of any of the foregoing. The excipients can be intragranular, intergranular, or mixtures thereof.

[0074] The vaccine composition can be formulated as freeze-dried or liquid preparations according to any means suitable in the art. Non-limiting examples of liquid form preparations include solutions, suspensions, syrups, slurries, and emulsions. Suitable liquid carriers include any suitable organic or inorganic solvent, for example, water, alcohol, saline solution, buffered saline solution, physiological saline solution, dextrose solution, water propylene glycol solutions, and the like, preferably in sterile form. After formulation, the vaccine composition can be incorporated into a sterile container which is then sealed and stored at a low temperature (e.g., 4°C), or it can be freeze dried.

[0075] The vaccine composition can be formulated in either neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the active polypeptides) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or organic acids such as acetic, oxalic, tartaric, mandelic, and the like. Salts formed from free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

[0076] The vaccine composition can optionally comprise agents that enhance the protective efficacy of the vaccine, such as adjuvants. Adjuvants include any compound or compounds that act to increase an immune response to the modified herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, or HSHV) gB protein, homotrimeric complex comprising the same, or protein complex comprising the homotrimer complex, thereby reducing the quantity of herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, or HSHV)

gB (or nucleic acid encoding the same) necessary in the vaccine, and/or the frequency of administration necessary to generate a protective immune response. Adjuvants can include for example, emulsifiers, muramyl dipeptides, avridine, aqueous adjuvants such as aluminum hydroxide, chitosan-based adjuvants, and any of the various saponins, oils, and other substances known in the art, such as Amphigen, LPS, bacterial cell wall extracts, bacterial DNA, CpG sequences, synthetic oligonucleotides and combinations thereof (Schijns *et al.* (2000) Curr. Opin. Immunol. 12:456), Mycobacterialphlei (*M. phlei*) cell wall extract (MCWE) (U.S. Patent No. 4,744,984), *M. phlei* DNA (M-DNA), and *M. phlei* cell wall complex (MCC). Compounds which can serve as emulsifiers include natural and synthetic emulsifying agents, as well as anionic, cationic and nonionic compounds. Among the synthetic compounds, anionic emulsifying agents include, for example, the potassium, sodium and ammonium salts of lauric and oleic acid, the calcium, magnesium and aluminum salts of fatty acids, and organic sulfonates such as sodium lauryl sulfate. Synthetic cationic agents include, for example, cetyltrimethylammonium bromide, while synthetic nonionic agents are exemplified by glycerylestere (e.g., glyceryl monostearate), polyoxyethylene glycol esters and ethers, and the sorbitan fatty acid esters (e.g., sorbitan monopalmitate) and their polyoxyethylene derivatives (e.g., polyoxyethylene sorbitan monopalmitate). Natural emulsifying agents include acacia, gelatin, lecithin and cholesterol.

[0077] Other suitable adjuvants can be formed with an oil component, such as a single oil, a mixture of oils, a water-in-oil emulsion, or an oil-in-water emulsion. The oil can be a mineral oil, a vegetable oil, or an animal oil. Mineral oils are liquid hydrocarbons obtained from petrolatum via a distillation technique, and are also referred to in the art as liquid paraffin, liquid petrolatum, or white mineral oil. Suitable animal oils include, for example, cod liver oil, halibut oil, menhaden oil, orange roughy oil and shark liver oil, all of which are available commercially. Suitable vegetable oils, include, for example, canola oil, almond oil, cottonseed oil, corn oil, olive oil, peanut oil, safflower oil, sesame oil, soybean oil, and the like. Freund's Complete Adjuvant (FCA) and Freund's Incomplete Adjuvant (FIA) are two common adjuvants that are commonly used in vaccine preparations, and are also suitable for use in the present invention. Both FCA and FIA are water-in-mineral oil emulsions; however, FCA also contains a killed *Mycobacterium sp.*

[0078] Immunomodulatory cytokines can also be used in the vaccine compositions to enhance vaccine efficacy, for example, as an adjuvant. Non-limiting examples of such cytokines include interferon alpha (IFN- α), interleukin-2 (IL-2), and granulocyte macrophage-colony stimulating factor (GM-CSF), or combinations thereof.

[0079] The vaccine composition can optionally further comprise other antigens from herpesviruses to further enhance the protective efficacy of the vaccine. In an embodiment, the additional herpesvirus antigens are derived from the same virus species as the modified gB protein. For example, if the vaccine composition comprises a modified HCMV gB protein, then the additional antigens are also HCMV antigens. In another non-limiting example, if the vaccine composition comprises a modified EBV gB protein, then the additional antigens are also EBV antigens. Non-limiting examples of such herpesvirus antigens include glycoprotein H (gH), glycoprotein L (gL), glycoprotein 350 (gp350), UL128, UL130, UL131, or combinations thereof. The nucleic acid and amino acid sequences of these herpesvirus antigens are known.

[0080] Any of the non-limiting other antigens can be multimerized according to PCT/US2013/052270. In an embodiment, the vaccine composition can include at least one, two, three, four, or up to five of the other antigens. In another embodiment, each of these antigens can be multimerized to create multimeric fusion proteins comprising multiple copies of a single antigen of interest (*e.g.*, a homodimer, homotrimer, or tetramer using two, three, or four copies of the same antigen), or to create multimeric fusion proteins comprising two or more different antigens of interest (*e.g.*, heterodimer, heterotrimer, tetramer, pentamer, hexamer, or octamer). Preferably, if the vaccine composition comprises a homotrimeric complex of HCMV gB, the vaccine composition also comprises a pentameric complex of HCMV gH/gL/UL128/UL130/UL131 or an HCMV gH/gL fusion protein with or without UL128/UL130/UL131. Also preferably, if the vaccine composition comprises a homotrimeric complex of EBV gB, the vaccine composition also comprises a tetramer of EBV gp350 and a monomer of an EBV gH/gL fusion protein.

[0081] In certain embodiments, the herpesvirus gH/gL fusion protein comprises a peptide linker sequence, as described herein, that joins the gH protein to the gL protein. In certain embodiments, the herpesvirus gH and gL proteins are from a herpesvirus selected from the group consisting of HCMV, HSV-1, HSV-2, VZV, EBV, and HSHV. The amino acid sequences of these herpesvirus gH and gL proteins are known. In one embodiment, the amino acid sequence of the HCMV gH/gL fusion protein comprises the sequence of SEQ ID NO: 25.

[0082] The vaccine composition can be prepared using techniques well known to those skilled in the art including, but not limited to, mixing, sonication and microfluidation. The adjuvant can comprise from about 10% to about 80% (v/v) of the vaccine composition,

more preferably about 20% to about 50% (v/v), and more preferably about 20% to about 30% (v/v), or any integer within these ranges.

[0083] The vaccine composition can be administered to any animal, and preferably is a mammal such as a human, mouse, rat, hamster, guinea pig, rabbit, cat, dog, monkey, cow, horse, pig, and the like. Humans are most preferred.

[0084] Administration of the vaccine composition can be by infusion or injection (e.g., intravenously, intramuscularly, intracutaneously, subcutaneously, intrathecal, intraduodenally, intraperitoneally, and the like). The vaccine composition can also be administered intranasally, vaginally, rectally, orally, intratonsillar, or transdermally. Additionally, the vaccine composition can be administered by “needle-free” delivery systems.

[0085] The effective amount of the vaccine composition may be dependent on any number of variables, including without limitation, the species, breed, size, height, weight, age, overall health of the patient, the type of formulation, or the mode or manner or administration. The appropriate effective amount can be routinely determined by those of skill in the art using routine optimization techniques and the skilled and informed judgment of the practitioner and other factors evident to those skilled in the art. Preferably, a therapeutically effective dose of the vaccine composition described herein will provide the therapeutic preventive benefit without causing substantial toxicity to the subject.

[0086] The vaccine composition can be administered to a patient on any schedule appropriate to induce and/or sustain an immune response against herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, or HSHV) gB or a herpesvirus protein complex comprising gB/gH/gL. For example, patients can be administered a vaccine composition as a primary immunization as described and exemplified herein, followed by administration of a secondary immunization, or booster, to bolster and/or maintain protective immunity.

[0087] The vaccine administration schedule, including primary immunization and booster administration, can continue as long as needed for the patient, for example, over the course of several years, to over the lifetime of the patient. The frequency of primary vaccine and booster administration and dose administered can be tailored and/or adjusted to meet the particular needs of individual patients, as determined by the administering physician according to any means suitable in the art.

[0088] The vaccine composition may be administered prophylactically (before exposure to the antigen or pathogen of interest) or therapeutically (after exposure to the antigen or pathogen of interest).

[0089] ***Methods of Inducing an Immune Response.*** In another aspect, a composition comprising 1) a homotrimer complex of the modified herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, or HSHV) gB protein (or nucleic acid encoding the modified herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, or HSHV) gB protein) or 2) a protein complex where the protein complex comprises a homotrimeric complex of a modified herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, or HSHV) gB protein and herpesvirus gH and gL proteins (e.g., a herpesvirus gH/gL fusion protein) can be used in a method of inducing an immune response. The immune response can be induced in a naïve subject who has not previously been exposed to HCMV or other herpesvirus. Alternatively, the immune response can be induced in a subject who has been previously exposed to a herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, or HSHV) and used to enhance an existing immune response.

[0090] In one embodiment, the method of enhancing an immune response comprises administering to a subject a composition comprising 1) a homotrimer complex of a modified herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, or HSHV) gB protein or 2) a protein complex where the protein complex comprises a homotrimeric complex of a modified herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, or HSHV) gB protein and herpesvirus gH and gL proteins (e.g., a herpesvirus gH/gL fusion protein), wherein the homotrimer complex of the herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, or HSHV) gB protein or the protein complex induces an immune response against HCMV or other herpesvirus. In another embodiment, the method of enhancing an immune response comprises administering to a subject a composition comprising a nucleic acid construct that encodes a modified herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, or HSHV) gB protein, as described in this application, wherein the modified herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, or HSHV) gB protein is expressed in the subject and a homotrimer complex thereof induces an immune response against the herpesvirus (e.g., HCMV, HSV-1, HSV-2, VZV, EBV, or HSHV) in the subject.

[0091] In these methods of inducing or suppressing an immune response, the immune response can be measured using routine methods in the art, such as those disclosed in this application. These routine methods include, but are not limited to, measuring an antibody response, such as an antibody response directed against a protein encoded by the recombinant vector, and measuring cellular proliferation, including, for example, by measuring tritiated thymidine incorporation or cytokine (e.g., IFN- γ) production.

[0092] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art. Although

methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. In case of conflict with any publication, patent application, patent, or other reference mentioned herein, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

EXAMPLES

Example 1 - Expression of trimeric HCMV gB protein

[0093] ***Construction of plasmids for production of trimeric HCMV gB.*** To test whether trimeric HCMV glycoprotein B can provide an effective and reproducible means for enhancing immune responses to HCMV infection, a recombinant nucleic acid plasmid (SEQ ID NO: 3) was designed to encode for amino acids 23-750 of SEQ ID NO: 2, with the coding sequence for the furin cleavage site (RTKRS (SEQ ID NO: 19) between amino acids 457-461 of SEQ ID NO: 2) being replaced with the coding sequence for a (Gly₄Ser)₃ (SEQ ID NO: 5) linker. Without intending to be bound by theory, it is believed that introduction of the (Gly₄Ser)₃ (SEQ ID NO: 5) linker allows for proper protein folding and thus formation of a homotrimeric HCMV glycoprotein B complex. The recombinant nucleic acid also included a nucleic acid encoding for an IgG κ leader sequence on the 5' end to direct protein secretion into the cell supernatant, and a nucleic acid encoding for a His₆ (SEQ ID NO: 26) sequence on the 3' end to aid in purification and immunohistochemical analysis. The recombinant nucleic acid (SEQ ID NO: 3) was cloned into the pOptiVEC vector (Life Technologies, Carlsbad, CA), and verified by sequencing.

[0094] ***Transfection of Chinese hamster ovary (CHO) cells (strain DG44).*** CHO DG44 cells were maintained in "CD DG44" medium (Life Technologies, Carlsbad, CA), and 2×10^7 cells were used for transfection. Thirty μ g of the recombinant nucleic acid construct was re-suspended in 1.2 ml OptiPro™ (Life Technologies, Carlsbad, CA) SFM medium after linearization with PvuI, followed by adding 30 μ l of FreeStyle™ Max Reagent (Life Technologies, Carlsbad, CA) mixed gently and incubated for 10 min at room temperature. The DNA-FreeStyle™ Max Reagent (Life Technologies, Carlsbad, CA) complex was slowly added into the flask containing 2×10^7 DG44 cells with gentle shaking. The cells were

incubated at 37°C, 5% CO₂ for 48 hours. Cells were centrifuged at 1,200 rpm and maintained in CD OptiCHO™ (Life Technologies, Carlsbad, CA) serum-free medium. Methotrexate (MTX, Sigma, St. Louis, MO) was used to select high recombinant protein-secreting cells, with the concentration of MTX gradually increased from 50 nM to 4 µM.

[0095] ***Immunohistochemical analysis of modified HCMV gB proteins with Anti-His Antibody.*** After MTX selection, modified HCMV gB expressing CHO cells were loaded into “Fibercell” cartridges (FiberCell Systems, Inc., Frederick, MD), and concentrated supernatants were collected daily. Supernatants were further concentrated by centrifugation at 3,000 rpm for 30 min using a Centriprep® Centrifugal Filter Unit, 30,000 MW cut-off (Thermo Scientific Fisher, Waltham, MA). Affinity purification was performed using a cobalt column (Thermo Scientific Fisher, Waltham, MA), according to manufacturer’s instructions. Briefly, concentrated supernatants were mixed with an equal volume of equilibration buffer, and added to the cobalt purification column. The column was incubated with gentle agitation for 60 min at 4°C and washed 3x with washing buffer. The modified HCMV gB proteins were eluted with elution buffer and analyzed by electrophoresis on 3-8% NuPAGE® Tris-Acetate Mini Gels (Life Technologies, Carlsbad, CA), under fully reducing or partially reducing conditions, and blotted with anti-His monoclonal antibody (Life Technologies, Carlsbad, CA).

[0096] Under fully reduced conditions (with sodium dodecyl sulfate (SDS), β-mercaptoethanol, and boiling for ten minutes), analysis by Western blot using anti-His antibody revealed a 120 kDa band, as shown in Figure 1A. This 120 kDa band is consistent with monomeric HCMV gB since fully reducing conditions disrupt any native oligomers into their monomeric form. These results demonstrate that in its non-native form, the modified HCMV gB of the present disclosure is a monomer.

[0097] Under partially reducing conditions (with sodium dodecyl sulfate (SDS), β-mercaptoethanol, and heating at 70°C for ten minutes), which allows for detection of HCMV gB in its native form, analysis by Western blot using anti-His antibody revealed a uniform band of higher molecular weight, approximately 360 kDa, as shown in Figure 1B. This band of about 360 kDa is consistent with the native, homotrimeric form of HCMV gB.

[0098] ***Immunohistochemical analysis of modified HCMV gB proteins with Anti-gB Antibody.*** The modified HCMV gB proteins were also analyzed by electrophoresis on 3-8% NuPAGE® Tris-Acetate Mini Gels (Life Technologies, Carlsbad, CA), under denaturing

or modified native conditions, and blotted with anti-CMV gB antibodies (2F12, Virusys, Taneytown, MD; or LS-C64457, LifeSpan BioSciences, Seattle, WA).

[0099] Under denaturing conditions, which disrupt any native oligomers into their monomeric form, modified HCMV gB was boiled for ten minutes in loading buffer containing 50 mM DTT. The proteins were then transferred to nitrocellulose membranes and blotted with anti-gB monoclonal antibodies (2F12, Virusys, Taneytown, MD; or LS-C64457, LifeSpan BioSciences, Seattle, WA). As shown in Figure 2B, the blots revealed a 120 kDa band corresponding with monomeric HCMV gB. These results demonstrate that the modified HCMV gB of the present disclosure in non-native form is a monomer.

[00100] Under modified native conditions, which allows for detection of HCMV glycoprotein B in its native form, modified HCMV gB was mixed with loading buffer containing LDS (lithium dodecyl sulfate) but no DTT and resolved in native running buffer. The proteins were then transferred to nitrocellulose membranes and blotted with anti-gB monoclonal antibody (LS-C64457, LifeSpan BioSciences, Seattle, WA). As shown in Figure 2A, the blots revealed a uniform band of about 360 kDa, which is consistent with the native, homotrimeric form of HCMV gB.

Example 2 – Immunization of mice with trimeric HCMC gB protein

[00101] ***Purified non-trimeric recombinant HCMV gB protein.*** A total of 2 mg of HCMV gB protein was purchased from Sino Biological, Inc. (Beijing, P.R. China). This HCMV gB protein was produced in the human embryonic kidney (HEK) 293 cell line using a DNA sequence encoding the extracellular domain (amino acids 1-700 of SEQ ID NO: 2) linked with the cytoplasmic domain (amino acids 777-907 of SEQ ID NO: 2), and fused with a polyhistidine tag at the C-terminal end to aid in protein purification. The furin cleavage site remained intact, but mutated so as to be ineffectual. This HCMV gB protein comprises 818 amino acids with a predicted molecular mass of 93 kDa under reducing conditions, but a molecular mass of 130-140 kDa due to glycosylation. The bioactivity of this protein was confirmed by its ability to bind biotinylated human CD209-Fc in a functional ELISA assay. Importantly, this HCMV gB protein is essentially identical to the non-trimeric HCMV gB protein used in clinical trials. (Pass et al., *N Engl J Med* 360: 1191-9).

[00102] ***Mice.*** Female BALB/c mice were purchased from the National Cancer Institute (Frederick, MD) and were used at 7-10 weeks of age for all protein immunizations. Female BALB/c mice were purchased from Harlan Laboratories (Indianapolis, IN) and were used at 4-6 weeks of age for all plasmid DNA vaccinations. These studies were conducted in

accordance with the principles set forth in the *Guide for Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, revised 1996), and were approved by the Uniformed Services University of the Health Sciences and the University of Washington Institutional Animal Care and Use Committees.

[00103] **Immunization.** Female BALB/c mice were immunized i.p. with 3 different doses (25, 5.0, and 1.0 µg/mouse) of a homotrimeric complex of modified HCMV gB or commercial non-trimeric HCMV gB protein. The homotrimeric or non-trimeric HCMV gB was adsorbed on 13 µg of alum adjuvant (Allhydrogel 2%, Brenntag Biosector, Denmark), and administered with or without 25 µg of a stimulatory 30 mer CpG-containing oligodeoxynucleotide (CpG-ODN). Serum samples for ELISA assays were obtained from blood taken from the tail vein on days 0, 14, 28, and 42 for measurement of serum titers of gB-specific IgG.

[00104] **Measurement of serum titers in mice of gB-specific IgG and IgG isotypes by ELISA.** Immulon 4 ELISA plates (Dyrex Technologies, Inc., Chantilly, VA) were coated (50 µL/well) with homotrimeric HCMV gB (5 µg/ml) in PBS overnight at 4°C. Plates were washed 3X with PBS + 0.1% Tween 20 and were blocked with PBS + 1% BSA for 1 h at 37°C. Threefold dilutions of serum samples from immunized mice, starting at a 1/50 serum dilution, in PBS + 1% BSA were added, incubated overnight at 4°C, and plates were washed 3X with PBS + 0.1% Tween 20. Alkaline phosphatase-conjugated polyclonal goat anti-mouse IgG, IgG3, IgG1, IgG2b, or IgG2a antibodies (SouthernBiotech, Birmingham, AL) (200 ng/ml final concentration) in PBS + 1% BSA were then added, and plates were incubated at 37°C for 1 h. Plates were then washed 5X with PBS + 0.1% Tween 20. Substrate (*p*-nitrophenyl phosphate, disodium; Sigma) at 1 mg/ml in TM buffer (1 M Tris + 0.3 mM MgCl₂, pH 9.8) was then added for color development. Color was read at an absorbance of 405 nm on a Multiskan Ascent® ELISA reader (Labsystems, Finland). The results are shown in Figure 4, demonstrating that a modified HCMV gB of this disclosure (“Trimer”) is markedly more immunogenic (significantly higher anti-HCMV gB IgG) than non-trimeric control HCMV gB (“Sino”).

[00105] **Measurement of serum gB-specific neutralizing antibody by competitive ELISA.** The competitive ELISA was adapted from that which we previously described (Colino J, Duke L, Arjunaraja S, Chen Q, Liu L, Lucas AH, Snapper CM. 2012. Differential Idiotypic Utilization for the In Vivo Type 14 Capsular Polysaccharide-Specific Ig Responses to Intact *Streptococcus pneumoniae* versus a Pneumococcal Conjugate Vaccine. *J Immunol*

189: 575-86). Briefly, inhibition mixtures will be prepared by mixing sera at varying dilutions with 10 µg/ml of HCMV gB protein with incubation for 24 h at 4°C, before being transferred to wells previously coated with 1 µg/ml of neutralizing mouse IgG1 anti-HCMV gB mAb LS-C64457 (LifeSpan BioSciences, Inc, Seattle, WA), and blocked with PBS-BSA. Sera from naïve mice or mice immunized with a control protein, such as EBV gp350, will serve as negative controls (i.e. no inhibition). In the final detection step, plates will be incubated with alkaline phosphatase-conjugated non-neutralizing mouse IgG1 anti-gB mAb 2F12 (Virusys, Taneytown, MD) for 1 h at 37°C followed by addition of substrate (*p*-nitrophenyl phosphate, disodium) added at 1 mg/ml in TM buffer for color development. Color will be read at an absorbance of 405 nm on a Multiskan Ascent® ELISA reader (Labsystems, Finland) until the OD405 nm for the standard wells reach predetermined values. A standard curve will be generated using known concentrations of neutralizing mouse IgG1 anti-HCMV gB mAb LS-C64457 in the inhibition mixtures to convert the OD405 nm of each serum sample into a final µg/ml concentration of gB-specific neutralizing antibody, using a four-parameter logistic regression method with correction for the serum dilution.

[00106] ***CMV neutralization assay.*** Neutralizing activities are determined by preparing 1:10 dilutions of each serum sample followed by additional 2-fold serial dilutions in culture medium. Each dilution is mixed with an equal volume of culture medium containing 4,000 pfu of HCMV (strain BADrUL131-Y4), incubated for 1h at 37°C then added to the wells of 384-well plates containing ARPE-19 (epithelial line, ATCC) or MRC-5 (fibroblast line, ATCC) monolayers. Each serum sample is assayed in triplicate and representative photomicrographs were taken using a Nikon Eclipse TS100 inverted UV microscope at four days post-infection. GFP fluorescence is measured seven days post-infection using a PerkinElmer Victor V1420 multilable counter. Fifty percent inhibitory concentration (IC₅₀) values and standard errors of the means are calculated using Prism software by plotting the means of triplicate GFP values for each serum dilution against log₂ serum concentration, calculating the best fit four-parameter equation for the data, and interpolating the serum dilution at the mid-point of the curve as the IC₅₀ neutralizing titer.

[00107] ***Statistics.*** All studies will be repeated at least 1x for reproducibility. Serum titers will be expressed as geometric means +/- standard error of the mean, with significance determined by a two-tailed students t-test ($p \leq 0.05$ considered significant). We previously determined that 7 mice per group give adequate statistical power to these studies.

Example 3 - Immunization of rabbits with trimeric HCMC gB protein

[00108] HCMV trimeric glycoprotein B (gB) induces highly boosted gB-specific IgG responses in rabbits that prevents *in vitro* HCMV infection of fibroblasts and epithelial cells. A group of 4 male New Zealand white rabbits, 12 to 15 weeks old were immunized subcutaneously with 25 ug of trimeric HCMV gB adsorbed to aluminum hydroxide (alum; 0.25 ug alum/mg protein). Rabbits were immunized on Day 0, Day 21, and Day 42 and serum samples were taken before initial immunization, and 10 days following each immunization. Serum titers of HCMV gB-specific IgG were determined. Primary immunization with trimeric HCMV gB elicited detectable serum titers of HCMV gB-specific IgG that were boosted about 100-fold following secondary immunization (Figure 5). A third immunization showed no further increases in serum titers.

[00109] *In vitro* neutralizing activity against live HCMV, using fibroblasts (MRC-5) and epithelial cells (ARPE-19) (Figure 6), was also analyzed. Human serum from a CMV-immune patient was used as a control (“human sera”). Induction of serum neutralizing titers from rabbits immunized with trimeric HCMV gB were observed and were comparable to those measured in human HCMV-immune sera, when assayed on fibroblasts (MRC-5) (Figure 6). Although serum neutralizing titers on epithelial cells (ARPE-19) were also observed in HCMV trimeric gB-immunized rabbits, they were significantly lower than that observed in the human HCMV-immune serum (Figure 6), suggesting a possible role for additional HCMV proteins in mediating protection on epithelial cells.

[00110] ***Measurement of serum titers in rabbits of gB-specific IgG isotypes by ELISA.*** Immulon 4 ELISA plates (Dynex Technologies, Chantilly, VA) were coated overnight with 5 µg/ml of HCMV gB protein in PBS (50 µl/well) at 4°C. The plates were then blocked with PBS + 1% bovine serum albumin (BSA) (100 µl/well) for 2 h at 37°C. Three-fold serial dilutions of serum samples, starting at a 1/50 serum dilution, in PBS plus 1% BSA (50 µl/well) were then added and incubated overnight at 4°C followed by washing (3x) with PBS + 0.1% Tween-20. Alkaline phosphatase-conjugated polyclonal goat anti-rabbit IgG Ab (Southern Biotechnology) (200 ng/ml, 50 µl/well) in PBS plus 1% BSA was then added and plates were incubated at 37°C for 1 h. Plates were then washed with PBS + 0.1% Tween-20 and substrate (p-nitrophenyl phosphate, disodium; Sigma-Aldrich) was added at 1 mg/ml in TM buffer (1 M Tris + 0.3 mM MgCl₂, pH 9.8) for color development.

Color was read at an absorbance of 405 nm on a Multiskan Ascent ELISA reader (Labsystems, Finland).

[00111] ***CMV neutralization assay.*** Neutralizing activities were determined by preparing 1:10 dilutions of each serum sample followed by additional 2-fold serial dilutions in culture medium. Each dilution was mixed with an equal volume of culture medium containing 4,000 pfu of HCMV (strain BADrUL131-Y4), incubated for 1h at 37°C then added to the wells of 384-well plates containing ARPE-19 (epithelial line, ATCC) or MRC-5 (fibroblast line, ATCC) monolayers. Each serum sample was assayed in triplicate and representative photomicrographs were taken using a Nikon Eclipse TS100 inverted UV microscope at four days post-infection. GFP fluorescence was measured seven days post-infection using a PerkinElmer Victor V1420 multilable counter. Fifty percent inhibitory concentration (IC₅₀) values and standard errors of the means were calculated using Prism software by plotting the means of triplicate GFP values for each serum dilution against log₂ serum concentration, calculating the best fit four-parameter equation for the data, and interpolating the serum dilution at the mid-point of the curve as the IC₅₀ neutralizing titer.

Example 4 - Expression of trimeric human EBV gB protein

[00112] ***Construction of plasmids for production of trimeric EBV gB.*** To test whether homotrimeric EBV glycoprotein B can provide an effective and reproducible means for enhancing immune responses to EBV infection, a recombinant nucleic acid plasmid (SEQ ID NO: 9) was designed to encode for amino acids 23-732 of SEQ ID NO: 8, with the coding sequence for the furin cleavage site (RRRRD (SEQ ID NO: 20) between amino acids 429-433 of SEQ ID NO: 8 being replaced with the coding sequence for a (Gly₄Ser)₃ (SEQ ID NO:5) linker (Figure 8). Without intending to be bound by theory, it is believed that introduction of the (Gly₄Ser)₃ linker allows for proper protein folding and thus formation of a trimeric EBV glycoprotein B complex. The EBV gB signal peptide (amino acids 1-22 of SEQ ID NO: 8) was replaced by an IgG κ leader sequence (SEQ ID NO:6). Thus, the recombinant nucleic acid further included a nucleic acid encoding for an IgG κ leader sequence on the 5' end to direct protein secretion into the cell supernatant, and a nucleic acid encoding for a His₆ (SEQ ID NO: 26) sequence on the 3' end to aid in purification and immunohistochemical analysis. The recombinant nucleic acid (SEQ ID NO: 9) was cloned into the pOptiVEC™ vector (Life Technologies, Carlsbad, CA), and verified by sequencing.

[00113] ***Transfection of Chinese hamster ovary (CHO) cells (strain DG44)*** CHO DG44 cells were maintained in “CD DG44” medium (Life Technologies, Carlsbad, CA), and

2×10^7 cells were used for transfection. 30 μ g of the recombinant nucleic acid construct were re-suspended in 1.2 ml OptiPro™ (Life Technologies, Carlsbad, CA) SFM medium after linearization with PvuI, followed by adding 30 μ l of FreeStyle Max Reagent™ (Life Technologies, Carlsbad, CA) mixed gently and incubated for 10 min at room temperature. The DNA-FreeStyle Max Reagent™ (Life Technologies, Carlsbad, CA) complex was slowly added into the flask containing 2×10^7 DG44 cells with gentle shaking. The cells were incubated at 37°C, 5% CO₂ for 48 hours. Cells were centrifuged at 1,200 rpm and maintained in CD OptiCHO™ (Life Technologies, Carlsbad, CA) serum-free medium. Methotrexate (MTX, Sigma, St. Louis, MO) was used to select high recombinant protein-secreting cells, with the concentration of MTX gradually increasing from 50 nM to 4 μ M.

[00114] ***Immunohistochemical analysis of modified EBV gB proteins with Anti-His Antibody.*** After MTX selection, modified EBV gB expressing CHO cells were loaded into “Fibercell” cartridges (FiberCell Systems, Inc., Frederick, MD), and concentrated supernatants were collected daily. Modified EBV gB expressing CHO cells were lysed with M-PER mammalian protein extraction reagent (Thermo Scientific Fisher, Waltham, MA), centrifuged at 3500 rpm for 60 min to remove cell debris. Supernatants were further concentrated by centrifugation at 3,000 rpm for 30 min using a Centriprep® Centrifugal Filter Unit (Thermo Scientific Fisher, Waltham, MA), 30,000 MW cut-off. Affinity purification was performed using a cobalt column (Thermo Scientific Fisher, Waltham, MA), according to manufacturer’s instructions. Briefly, concentrated supernatants were mixed with an equal volume of equilibration buffer, and added to the cobalt purification column. The column was incubated with gentle agitation for 60 min at 4°C and washed 3x with washing buffer.

[00115] ***Immunohistochemical analysis of modified EBV gB proteins with Anti-EBV gB Antibody.*** The modified EBV gB proteins were analyzed by electrophoresis on 3-8% NuPAGE® Tris-Acetate Mini Gels (Life Technologies, Carlsbad, CA), under denaturing or modified native conditions, and blotted with anti-His monoclonal antibody (Life Technologies, Carlsbad, CA) and anti-EBV gB antibodies (Virusys, Taneytown, MD).

[00116] Under denaturing conditions, which disrupt any native oligomers into their monomeric form, modified (“trimeric”) EBV gB was boiled for ten minutes in loading buffer containing 50 mM DTT. The proteins were then transferred to nitrocellulose membranes and blotted with anti-His monoclonal antibody ((Life Technologies, Carlsbad, CA) or anti-gB monoclonal antibodies (Virusys, Taneytown, MD). As shown in Figure 7A, the blots

revealed an 80 kDa band corresponding with monomeric EBV gB. These results demonstrate that the modified EBV gB in non-native form is a monomer.

[00117] Under modified native conditions, which allows for detection of EBV gB in its native form, modified EBV gB was mixed with loading buffer containing LDS (lithium dodecyl sulfate) but no DTT and resolved in native running buffer. The proteins were then transferred to nitrocellulose membranes and blotted with anti-His monoclonal antibody (Figure 7B) or anti-gB monoclonal antibody (Figure 7C). As shown in Figures 7B/C, the blots revealed a uniform band of about 240 kDa, which is consistent with the native, trimeric form of EBV gB.

Example 5 – Immunization studies with trimeric human EBV gB protein

[00118] **Mice.** Female BALB/c mice will be purchased from the National Cancer Institute (Frederick, MD) and will be used at 7-10 weeks of age for all protein immunizations. Female BALB/c mice will be purchased from Harlan Laboratories (Indianapolis, IN) and will be used at 4-6 weeks of age for all plasmid DNA vaccinations. These studies will be conducted in accordance with the principles set forth in the *Guide for Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, revised 1996), and will be approved by the Uniformed Services University of the Health Sciences and the University of Washington Institutional Animal Care and Use Committees.

[00119] **Immunizations.** Female BALB/c mice will be immunized i.p. with 3 different doses (25, 5.0, and 1.0 µg/mouse) of a homotrimeric complex of modified EBV gB or non-trimeric EBV gB protein. The homotrimeric or non-trimeric EBV gB will be adsorbed on 13 µg of alum adjuvant (Allhydrogel 2%, Brenntag Biosector, Denmark), and administered with or without 25 µg of a stimulatory 30 mer CpG-containing oligodeoxynucleotide (CpG-ODN). Serum samples for ELISA assays will be obtained from blood taken from the tail vein on days 0, 14, 28, and 42 for measurement of serum titers of gB-specific IgG.

[00120] **Measurement of serum titers of gB-specific IgG and IgG isotypes by ELISA.** Immulon 4 ELISA plates (Dynex Technologies, Inc., Chantilly, VA) will be coated (50 µL/well) with homotrimeric EBV gB (5 µg/ml) in PBS overnight at 4°C. Plates will be washed 3X with PBS + 0.1% Tween 20 and will be blocked with PBS + 1% BSA for 1 h at 37°C. Threefold dilutions of serum samples from immunized mice, starting at a 1/50 serum dilution, in PBS + 1% BSA will be added, incubated overnight at 4°C, and plates will be

washed 3X with PBS + 0.1% Tween 20. Alkaline phosphatase-conjugated polyclonal goat anti-mouse IgG, IgG3, IgG1, IgG2b, or IgG2a antibodies (SouthernBiotech, Birmingham, AL) (200 ng/ml final concentration) in PBS + 1% BSA will then be added, and plates will be incubated at 37°C for 1 h. Plates will then be washed 5X with PBS + 0.1% Tween 20. Substrate (*p*-nitrophenyl phosphate, disodium; Sigma) at 1 mg/ml in TM buffer (1 M Tris + 0.3 mM MgCl₂, pH 9.8) will then be added for color development. Color will be read at an absorbance of 405 nm on a Multiskan Ascent® ELISA reader (Labsystems, Finland).

[00121] ***EBV neutralization assay.*** The method developed in Dr. Jeffery Cohen's Lab at NIH will be used (Sashihara J et al, Virology 2009, 391: 249-256). Briefly, serum samples will be serially diluted in 2-fold steps (from undiluted to 18 serial dilutions) and 25 µL of the diluted sample or control antibody will be added to wells of a 96 well plate in triplicate. 25 µL of B95-8/F EBV virus will then be added to each well and incubated for 2 hrs. 50 µL of 1×10^5 Raji cells will be added and incubated for 1 hour at 37°C, the cells will be washed twice by centrifuging the plates at $300 \times g$ for 5 min and replacing the media, and incubated for 3 days at 37°C. The plate will then be centrifuged, the cells be washed once with PBS, and be fixed in 2% paraformaldehyde in PBS.

[00122] GFP-expressing cells will be quantified using a FACSCalibur™ Flow Cytometer (BD Biosciences, San Jose, CA, USA) and FlowJo software (Tree Star Inc., Ashland, OR). The effective dilution of antibody that inhibited infectivity by 50% (EDI50) based on reduction of the number of GFP positive cells will be calculated by non-linear regression analysis using GraphPad PRISM® software (GraphPad Software, La Jolla, CA).

[00123] ***Statistics.*** All studies will be repeated at least 1x for reproducibility. Serum titers will be expressed as geometric means +/- standard error of the mean, with significance determined by a two-tailed students t-test ($p \leq 0.05$ considered significant). We previously determined that 7 mice per group give adequate statistical power to these studies.

Example 6 - Expression of HCMV gH/gL fusion protein

[00124] The HCMV gH/gL heterodimer is part of the herpesvirus family core fusion machinery that is necessary for HCMV fusion and penetration into fibroblasts cells, epithelial cells, endothelial cells, and dendritic cells. Vaccination of rabbits with recombinant gH/gL alone elicited neutralizing antibodies against fibroblasts and epithelial cells, although neutralization was somewhat higher against epithelial cells, when using the entire pentameric complex (gH/gL/UL128/130/131A) (66).

[00125] The coding sequences for HCMV gH and gL were downloaded from NCBI, reference sequence NC_006273.2, version GI:155573622, including gH nucleotides 109224 through 111452 (SEQ ID NO: 21), gL nucleotides 165022 through 165858 (SEQ ID NO: 22). The construct for a herpesvirus gH/gL fusion protein was designed using MacVector. The amino acid sequences of wild type HCMV gH (SEQ ID NO: 18) and HCMV gL (SEQ ID NO: 24) are known. A nucleic acid encoding amino acids 31-278 of wild type HCMV gL was used (SEQ ID NO: 23), and the signal peptide 1-30 was replaced with an IgG κ leader sequence (SEQ ID NO:6). A nucleic acid encoding amino acids 24-718 amino acids of wild type HCMV gH was used (SEQ ID NO: 17) and linked to the 3' end of gL, separated by a 15 amino acid linker (Gly₄Ser)₃ sequence (SEQ ID NO:5), and a His6 (SEQ ID NO: 26) coding sequence was linked to the 3' end of gH for protein purification. The amino acid sequence of the gH/gL construct corresponds to SEQ ID NO: 25. DNA coding for the gH/gL was synthesized by Blue Heron Biotechnology, Inc, cloned into pOptiVEC™ (Invitrogen), and verified by sequencing. Chinese Hamster Ovary cells (strain DG44) (Invitrogen) were transfected with pOptiVEC™-gH/gL constructs using Free-style™ Max reagent (Invitrogen), and selected with gradually increased concentration of methotrexate up to 4 μ M. Supernatants were concentrated and purified using Cobalt affinity purification (Thermo Scientific), and analyzed by Western blot using both an anti-His6 (SEQ ID NO: 26) antibody and anti HCMV gH/gL antibody (Santa Cruz Biotech). Under reducing conditions, the Western blot demonstrated monomeric gH/gL as a 110 KDa band with either a monoclonal anti-His antibody (Figure 9A) or a monoclonal anti-gH antibody (Figure 9B).

Example 7 - Production of HCMV protein complex gB/gH/gL

[00126] HCMV entry into fibroblasts requires an HCMV envelope complex of trimeric gB, gH, and gL proteins, whereas the additional complexing of UL128/130/131A to gH/gL, in association with gB, is required for entry into endothelial, epithelial, and dendritic cells, and leukocytes (4, 5, 6).

[00127] Purified HCMV trimeric gB, as produced in Example 1, was mixed with purified monomeric gH/gL, as produced in Example 6, at a molecular ratio of 1:1, and incubated at room temperature for 2 hours. Subsequent analysis by Western blot under non-reducing conditions demonstrated a protein complex with a molecular weight of about 600 kDa (Figure 10), consistent with a complex of one HCMV trimeric gB and two HCMV monomeric gH/gL heterodimers. There have been no reports demonstrating that these viral

proteins, in their natural conformation, assemble into a native complex upon *in vitro* co-incubation. This may be due, in part, to the fact that it was previously not possible to produce a fully trimeric HCMV gB protein, which represents the HCMV gB in its natural conformation. This natural complex of HCMV proteins, which has not been previously expressed *in vitro*, represents a breakthrough in the design of prophylactic vaccines.

[00128] This protein complex vaccine also has implications beyond herpesvirus vaccines, as the same principle can be used to reconstitute protein complexes from the individual proteins of other viral or bacterial pathogens, which can, in turn, be used as vaccines to induce highly efficient neutralizing antibodies against conformational epitopes in the protein complex.

Example 8 – Immunization studies with HCMV protein complex gB/gH/gL

[00129] **Mice.** Female BALB/c mice will be purchased from the National Cancer Institute (Frederick, MD) and will be used at 7-10 weeks of age for all protein immunizations. Female BALB/c mice will be purchased from Harlan Laboratories (Indianapolis, IN) and will be used at 4-6 weeks of age for all plasmid DNA vaccinations. These studies will be conducted in accordance with the principles set forth in the *Guide for Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, revised 1996), and will be approved by the Uniformed Services University of the Health Sciences and the University of Washington Institutional Animal Care and Use Committees.

[00130] **Immunizations.** Female BALB/c mice will be immunized i.p. with 3 different doses (25, 5.0, and 1.0 µg/mouse) of a HCMV gB/gH/gL protein complex as produced in Example 6. The HCMV gB/gH/gL protein complex will be adsorbed on 13 µg of alum adjuvant (Allhydrogel 2%, Brenntag Biosector, Denmark), and administered with or without 25 µg of a stimulatory 30 mer CpG-containing oligodeoxynucleotide (CpG-ODN). Serum samples for ELISA assays will be obtained from blood taken from the tail vein on days 0, 14, 28, and 42 for measurement of serum titers of gB, gH, and/or gL specific IgG.

[00131] **Measurement of serum titers of gB/gH/gL-specific IgG and IgG isotypes by ELISA.** Immulon 4 ELISA plates (Dynex Technologies, Inc., Chantilly, VA) will be coated (50 µL/well) with HCMV gB/gH/gL protein complex (5 µg/ml) in PBS overnight at 4°C. Plates will be washed 3X with PBS + 0.1% Tween 20 and will be blocked with PBS + 1% BSA for 1 h at 37°C. Threefold dilutions of serum samples from immunized mice, starting at

a 1/50 serum dilution, in PBS + 1% BSA will be added, incubated overnight at 4°C, and plates will be washed 3X with PBS + 0.1% Tween 20. Alkaline phosphatase-conjugated polyclonal goat anti-mouse IgG, IgG3, IgG1, IgG2b, or IgG2a antibodies (SouthernBiotech, Birmingham, AL) (200 ng/ml final concentration) in PBS + 1% BSA will then be added, and plates will be incubated at 37°C for 1 h. Plates will then be washed 5X with PBS + 0.1% Tween 20. Substrate (*p*-nitrophenyl phosphate, disodium; Sigma) at 1 mg/ml in TM buffer (1 M Tris + 0.3 mM MgCl₂, pH 9.8) will then be added for color development. Color will be read at an absorbance of 405 nm on a Multiskan Ascent® ELISA reader (Labsystems, Finland).

[00132] **CMV neutralization assay.** Neutralizing activities are determined by preparing 1:10 dilutions of each serum sample followed by additional 2-fold serial dilutions in culture medium. Each dilution is mixed with an equal volume of culture medium containing 4,000 pfu of HCMV (strain BADrUL131-Y4), incubated for 1h at 37°C then added to the wells of 384-well plates containing ARPE-19 (epithelial line, ATCC) or MRC-5 (fibroblast line, ATCC) monolayers. Each serum sample is assayed in triplicate and representative photomicrographs were taken using a Nikon Eclipse TS100 inverted UV microscope at four days post-infection. GFP fluorescence is measured seven days post-infection using a PerkinElmer Victor V1420 Multilable Counter. Fifty percent inhibitory concentration (IC₅₀) values and standard errors of the means are calculated using Prism software by plotting the means of triplicate GFP values for each serum dilution against log₂ serum concentration, calculating the best fit four-parameter equation for the data, and interpolating the serum dilution at the mid-point of the curve as the IC₅₀ neutralizing titer.

[00133] **Statistics.** All studies will be repeated at least 1x for reproducibility. Serum titers will be expressed as geometric means +/- standard error of the mean, with significance determined by a two-tailed students t-test ($p \leq 0.05$ considered significant). We previously determined that 7 mice per group give adequate statistical power to these studies.

[00134] The following references are cited in the application and provide general information on the field of the invention and provide assays and other details discussed in the application.

1. Spaete RR. 1991. A recombinant subunit vaccine approach to HCMV vaccine development. *Transplant Proc* 23: 90-6

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4. Hahn G, Revello MG, Patrone M, Percivalle E, Campanini G, Sarasini A, Wagner M, Gallina A, Milanesi G, Koszinowski U, Baldanti F, Gerna G. 2004. Human cytomegalovirus UL131-128 genes are indispensable for virus growth in endothelial cells and virus transfer to leukocytes. *J Virol* 78: 10023-33
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6. Gerna G, Percivalle E, Lilleri D, Lozza L, Fornara C, Hahn G, Baldanti F, Revello MG. 2005. Dendritic-cell infection by human cytomegalovirus is restricted to strains carrying functional UL131-128 genes and mediates efficient viral antigen presentation to CD8⁺ T cells. *J Gen Virol* 86: 275-84

WHAT IS CLAIMED:

1. A human herpesvirus glycoprotein B (gB) polypeptide that is modified to enable production of a homotrimeric complex, comprising a modified extracellular domain or a fragment of the modified extracellular domain, wherein the modified extracellular domain or the fragment of the modified extracellular domain comprises a peptide linker inserted into the furin cleavage site, wherein the peptide linker is 5 to about 70 amino acids in length and wherein expression of the modified human herpesvirus gB polypeptide results in the formation of a homotrimeric complex that provides enhanced immunity to a herpesvirus infection.
2. The human herpesvirus gB polypeptide of claim 1, wherein the human herpesvirus gB is selected from the group consisting of human cytomegalovirus (HCMV) gB, VZV (Varicella-Zoster Virus) gB, EBV (Epstein-Barr Virus) gB, and HSHV (Kaposi Sarcoma-related Herpes Virus) gB.
3. The human herpesvirus gB polypeptide of claim 2, wherein the human herpesvirus gB is HCMV gB or EBV gB.
4. The human herpesvirus gB polypeptide of any one of claims 1-3, wherein the human herpesvirus gB is HCMV gB and wherein the modified extracellular domain comprises a peptide linker sequence inserted into the furin cleavage site at amino acids 457-461 of the wild type HCMV amino acid sequence (SEQ ID NO: 2).
5. The human herpesvirus gB polypeptide of any one of claims 1-4, wherein the human herpesvirus gB polypeptide does not include a transmembrane domain or an intracellular domain, which together have the amino acid sequence of amino acids 751-906 of SEQ ID NO:2.

6. The human herpesvirus gB polypeptide of any one of claims 1-3, wherein the human herpesvirus gB is EBV gB and wherein the modified extracellular domain comprises a peptide linker sequence inserted into the furin cleavage site at amino acids 427-434 of the wild type EBV amino acid sequence (SEQ ID NO: 8).
7. The human herpesvirus gB polypeptide of any one of claims 1-3, wherein the amino acid sequence of the human herpesvirus gB polypeptide comprises SEQ ID NO: 4.
8. The human herpesvirus gB polypeptide of any one of claims 1-7, wherein the peptide linker is about 15 amino acids in length.
9. The human herpesvirus gB polypeptide of any one of claims 1-8, wherein the peptide linker consists of the amino acid sequence GGGGSGGGGSGGGGS (SEQ ID NO: 5).
10. The human herpesvirus gB polypeptide of any one of claims 1-9, further comprising a leader sequence at the N-terminus of the gB polypeptide, wherein the leader sequence is not the native gB polypeptide leader sequence.
11. The human herpesvirus gB polypeptide of claim 10, wherein the leader sequence has the amino acid sequence METDTLLLWVLLLWVPGSTGD (SEQ ID NO: 6).
12. A herpesvirus gB polypeptide homotrimer complex, wherein the homotrimer complex comprises three human herpesvirus gB polypeptides of any one of claims 1-11.
13. A herpesvirus gB polypeptide homotrimer complex, wherein the homotrimer complex comprises three human herpesvirus gB polypeptides of any one of the claims 1-5 and 7, wherein the herpesvirus is HCMV and the homotrimer complex has a molecular weight (MW) of about 360 kDa.
14. A vaccine composition comprising the herpesvirus gB polypeptide homotrimer complex of claim 12 or 13 and a pharmaceutically acceptable excipient.
15. The vaccine composition of claim 14, further comprising at least one human herpesvirus antigen.

16. The vaccine composition of claim 15, wherein the at least one human herpesvirus antigen is selected from the group consisting of glycoprotein H (gH), glycoprotein L (gL), glycoprotein 350 (gp350), UL128, UL130, UL131, and combinations thereof.
17. The vaccine composition of claim 15 or 16, wherein the at least one human herpesvirus antigen is a multimer.
18. The vaccine composition of any one of claims 14-17, further comprising an adjuvant.
19. The vaccine composition of any one of claims 14-18, wherein at least 70% of the human herpesvirus gB polypeptides are homotrimers.
20. A protein complex that induces an immune response following administration to a subject, the protein complex comprising a herpesvirus gB polypeptide homotrimer complex, a herpesvirus glycoprotein H (gH), and a glycoprotein L (gL), wherein the homotrimer complex comprises three human herpesvirus gB polypeptides and each human herpesvirus glycoprotein B (gB) polypeptide comprises a modified extracellular domain or a fragment of the modified extracellular domain, wherein the modified extracellular domain or fragment of the modified extracellular domain comprises a peptide linker inserted into the furin cleavage site, wherein the peptide linker is from 5 to about 70 amino acids in length.
21. The protein complex of claim 20, wherein the herpesvirus gH and the herpesvirus gL form a herpesvirus gH/gL fusion protein.
22. The protein complex of claim 21, wherein the herpesvirus gH/gL fusion protein comprises the amino acid sequence of SEQ ID NO:25.
23. The protein complex of any one of claims 20-22, wherein the human herpesvirus gB is selected from the group consisting of human cytomegalovirus (HCMV) gB, VZV (Varicella-Zoster Virus) gB, EBV (Epstein-Barr Virus) gB, and HSHV (Kaposi Sarcoma-related Herpes Virus) gB.

24. The protein complex of any one of claims 20-23, wherein the human herpesvirus gB is HCMV gB or EBV gB.
25. The protein complex of any one of claims 20-24, wherein the human herpesvirus gB is HCMV gB and wherein the modified extracellular domain comprises a peptide linker sequence inserted into the furin cleavage site at amino acids 457-461 of the wild type HCMV amino acid sequence (SEQ ID NO: 2).
26. The protein complex of any one of claims 20-25, wherein the human herpesvirus gB polypeptide does not include a transmembrane domain or an intracellular domain, which together have the amino acid sequence of amino acids 751-906 of SEQ ID NO: 2.
27. The protein complex of any one of claims 20-24, wherein the human herpesvirus gB is EBV gB and wherein the modified extracellular domain comprises a peptide linker sequence inserted into the furin cleavage site at amino acids 427-434 of the wild type EBV amino acid sequence (SEQ ID NO: 8).
28. The protein complex of any one of claims 20-27, wherein the peptide linker is about 15 amino acids in length.
29. The protein complex of any one of claims 20-28, wherein the peptide linker consists of the amino acid sequence GGGGSGGGGSGGGGS (SEQ ID NO: 5).
30. The protein complex of any one of claims 20-29, wherein the human herpesvirus gB polypeptide further comprises a leader sequence at the N-terminus of the gB polypeptide, wherein the leader sequence is not the native gB polypeptide leader sequence.
31. The protein complex of claim 30, wherein the leader sequence has the amino acid sequence METDTLLLWVLLLWVPGSTGD (SEQ ID NO: 6).

32. The protein complex of any one of claims 20-31, wherein the protein complex further comprises a herpesvirus UL128, UL130, and UL131 polypeptide.
33. A vaccine composition comprising the protein complex of any one of claims 20-32 and a pharmaceutically acceptable excipient.
34. The vaccine composition of claim 33, further comprising an adjuvant.
35. Use of a human herpesvirus gB polypeptide of any one of claims 1-11, a herpesvirus gB polypeptide homotrimer complex of claim 12 or 13, a vaccine composition of any one of claims 14-19 and 33-34, or a protein complex of any one of claims 20-32 for the prevention and/or treatment of herpesvirus infection in a patient.
36. Use of a human herpesvirus gB polypeptide of any one of claims 1-11, a herpesvirus gB polypeptide homotrimer complex of claim 12 or 13, a vaccine composition of any one of claims 14-19 and 33-34, or a protein complex of any one of claims 20-32 for inducing immunity to herpesvirus infection in a patient.
37. A nucleic acid that encodes the human herpesvirus gB polypeptide of any one of claims 1-11.
38. A recombinant vector comprising the nucleic acid of claim 37.
39. Use of a human herpesvirus gB polypeptide of any one of claims 1-11, a herpesvirus gB polypeptide homotrimer complex of claim 12 or 13, a vaccine composition of any one of claims 14-19 and 33-34, or a protein complex of any one of claims 20-32 for the preparation of a medicament for preventing or treating herpesvirus infection in a patient.
40. Use a human herpesvirus gB polypeptide of any one of claims 1-11, a herpesvirus gB polypeptide homotrimer complex of claim 12 or 13, a vaccine composition of any one of claims 14-19 and 33-34, or a protein complex of any one of claims 20-32 for the

preparation of a medicament for inducing immunity to herpesvirus infection in a subject.

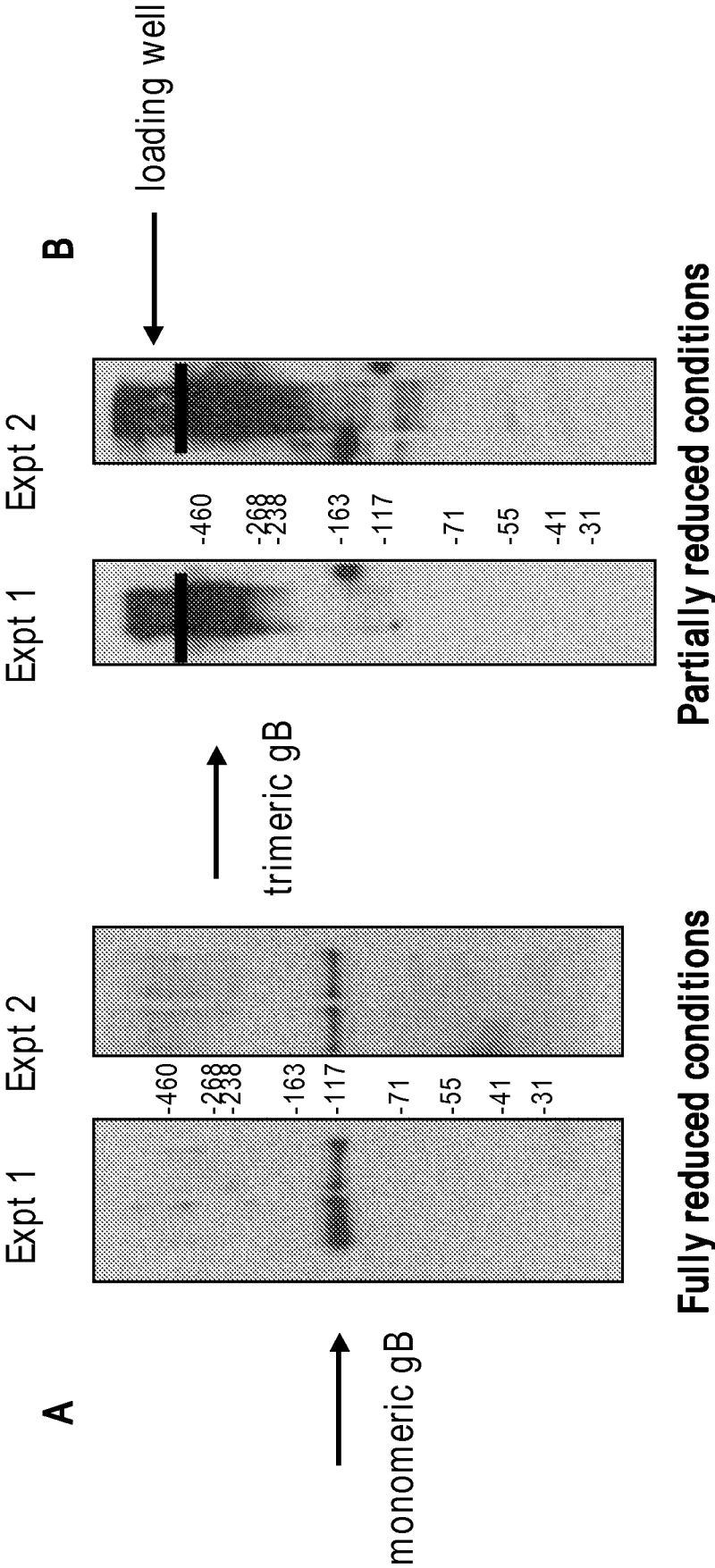
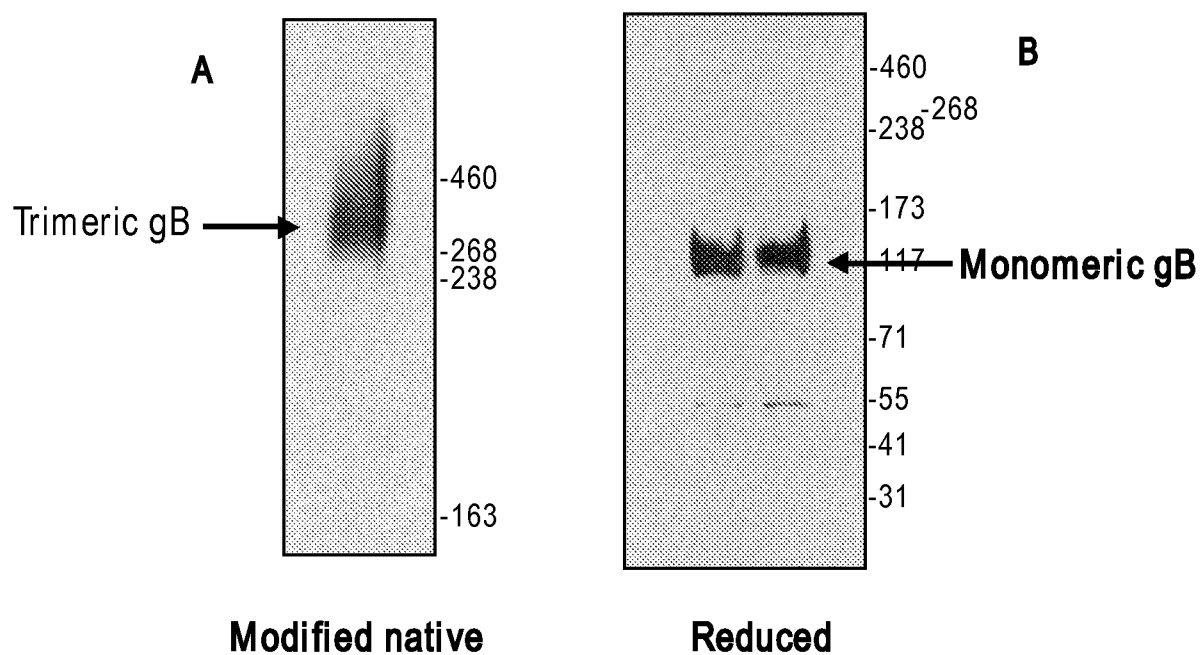
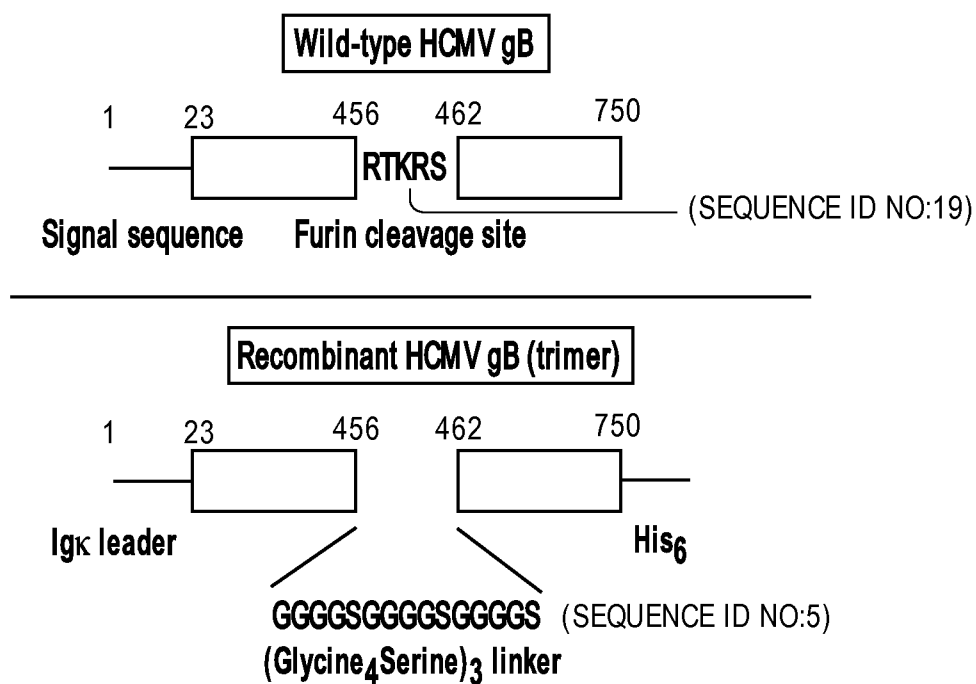
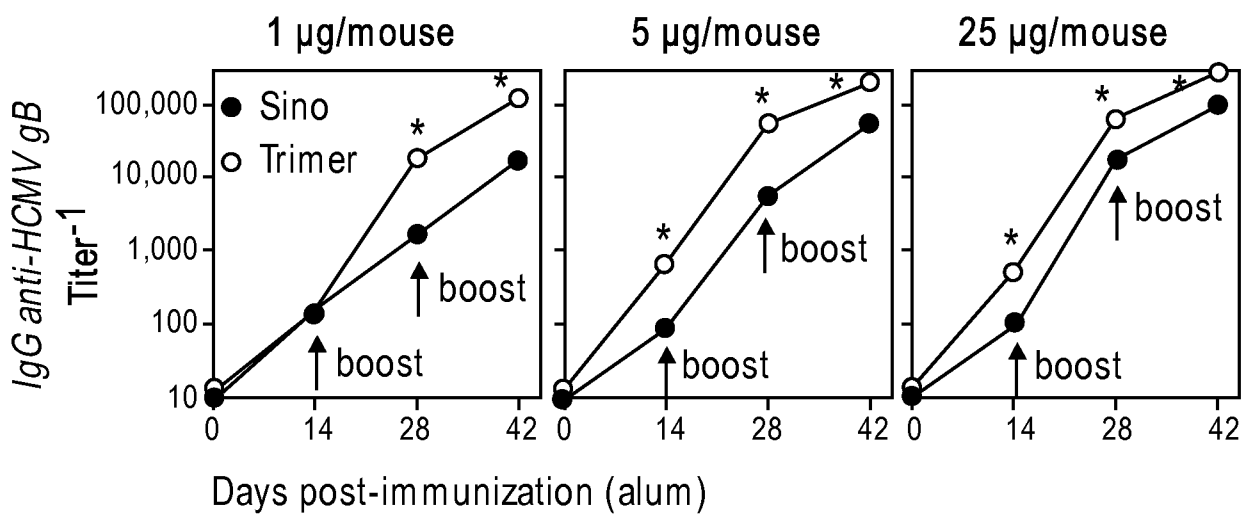


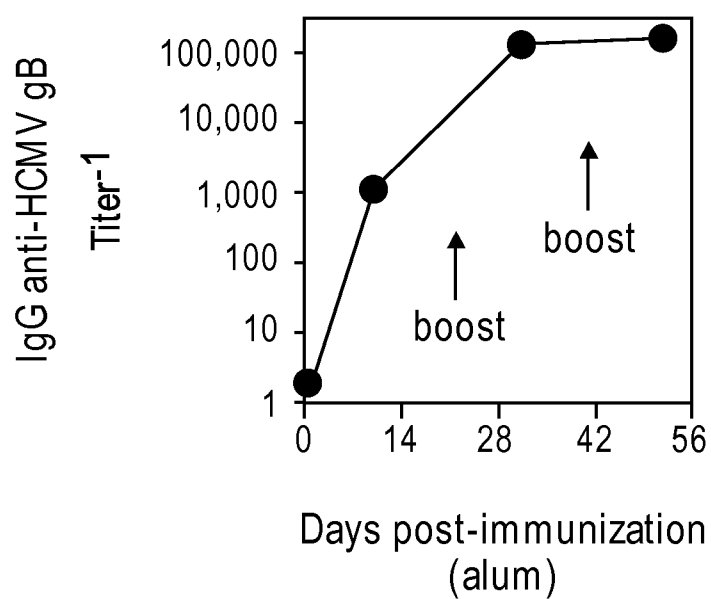
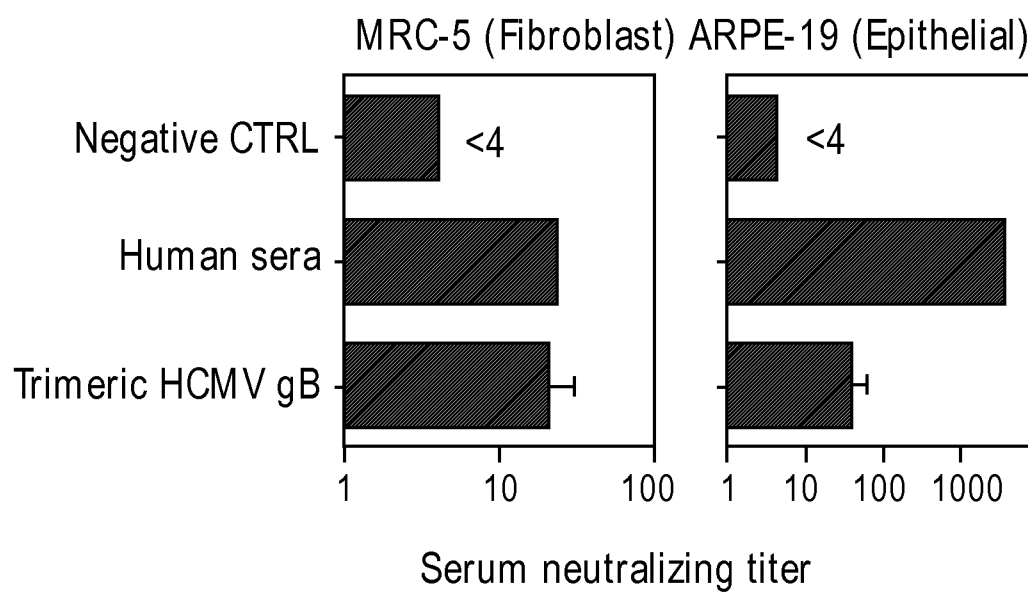
FIG. 1

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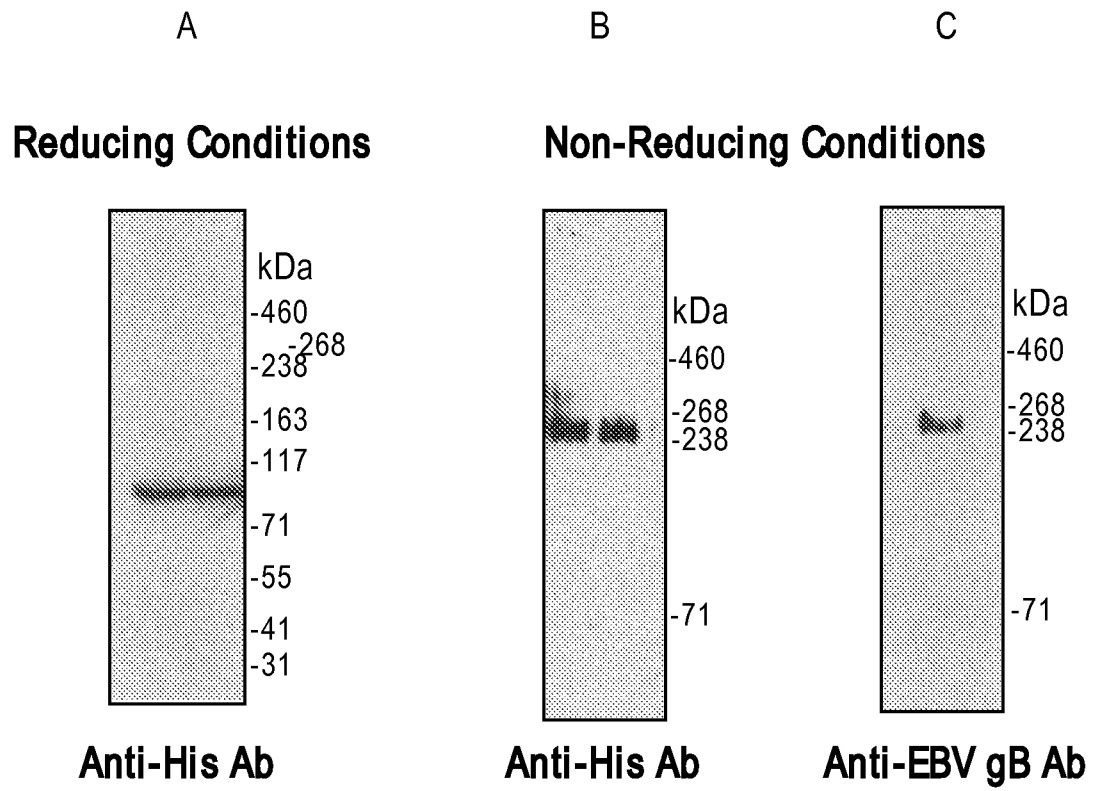
**FIG. 2****FIG. 3**



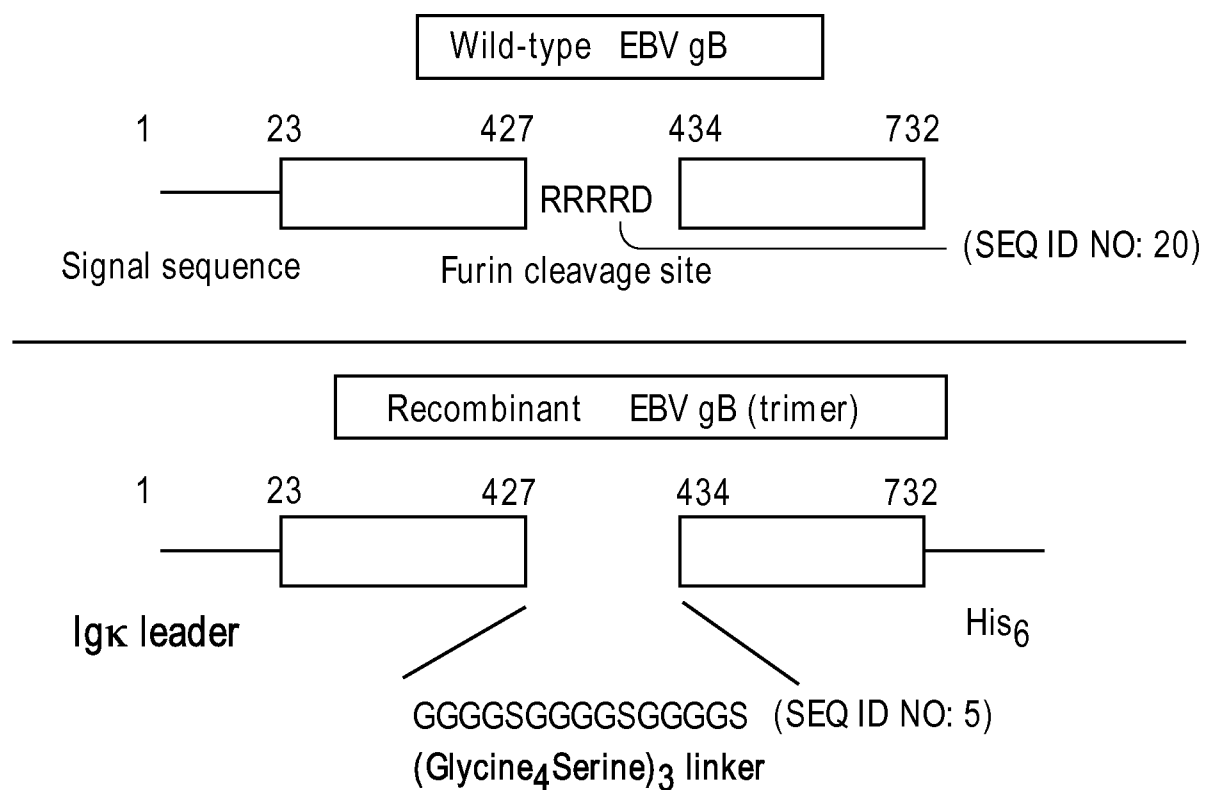
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**FIG. 5****FIG. 6**

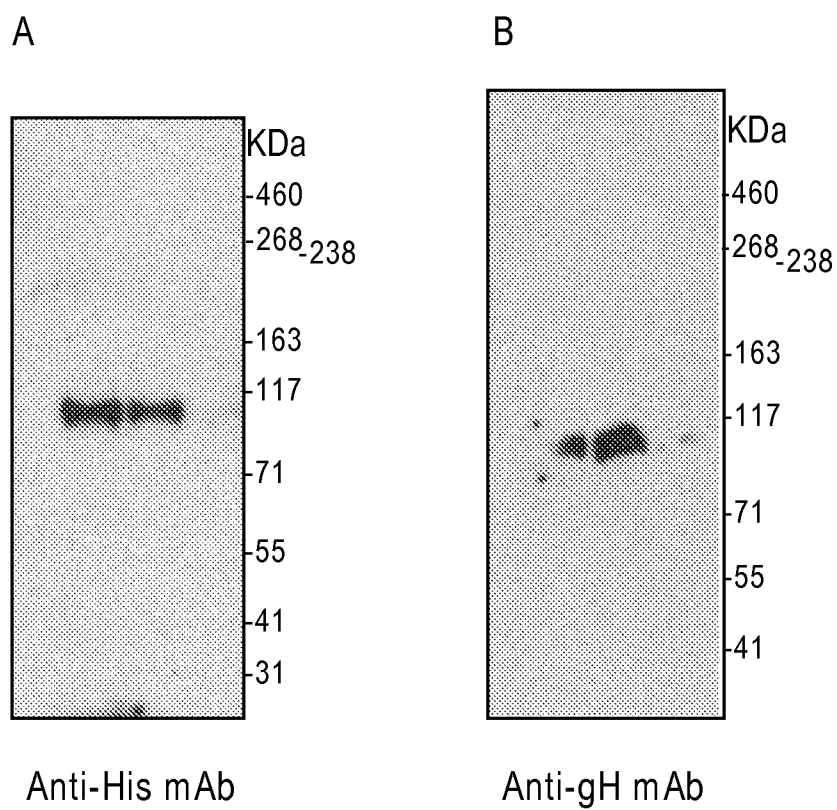
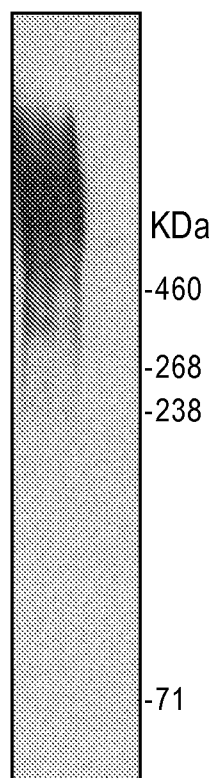
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**FIG. 7**

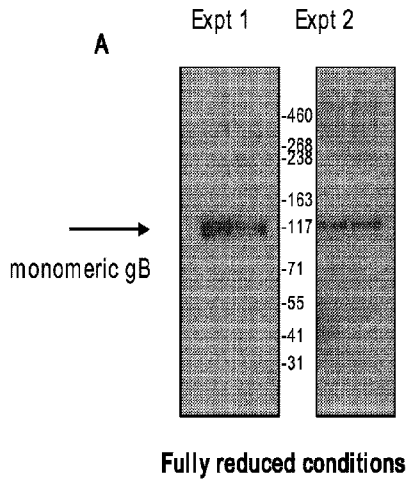
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**FIG. 8**

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**FIG. 9****FIG. 10**

A



→
trimeric gB

