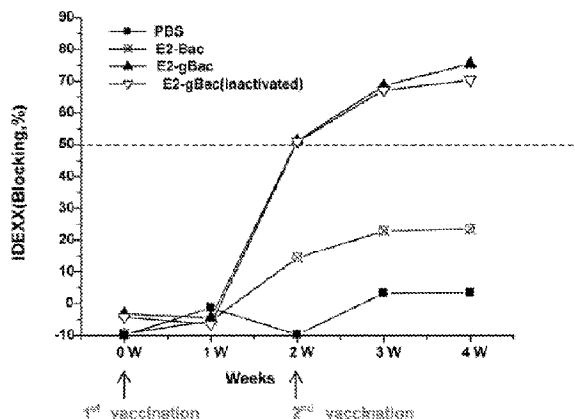




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SN titer	IDXX(Blocking,%)
<2	19 ± 1
2	24 ± 2
4	30 ± 2
8	36 ± 2
16	43 ± 2
32	49 ± 2
64	56 ± 2
128	62 ± 2
256	68 ± 2
512	75 ± 2
1024	80 ± 1

(57) Abrégé/Abstract:

A recombinant baculovirus displaying on its envelop a fusion protein is disclosed. The fusion protein comprises a heterologous antigen, and a C-terminal region of baculovirus envelope GP64 protein, which has at least 100 amino acid residues in length and lacks a B12D5 binding epitope located within the central basic region of the GP64 protein. The genome of the recombinant baculovirus comprises a transgene encoding a fusion protein that comprises a signal peptide, the heterologous antigen, and the C-terminal region of the baculovirus envelope GP64 protein, in which the antigen is located between the signal peptide and the C-terminal region of the GP64 protein. The recombinant baculovirus is for use in eliciting an antigen-specific immunogenic response in a subject in need thereof.

ABSTRACT

A recombinant baculovirus displaying on its envelope a fusion protein is disclosed. The fusion protein comprises a heterologous antigen, and a C-terminal region of baculovirus envelope GP64 protein, which comprises at least 100 amino acid residues in length and lacks a B12D5 binding epitope located within the central basic region of the GP64 protein. The genome of the recombinant baculovirus comprises a transgene encoding a fusion protein that comprises a signal peptide, the heterologous antigen, and the C-terminal region of the baculovirus envelope GP64 protein, in which the antigen is located between the signal peptide and the C-terminal region of the GP64 protein. The recombinant baculovirus is for use in eliciting an antigen-specific immunogenic response in a subject in need thereof.

NOVEL BACULOVIRUS VECTORS AND METHODS OF USE

FIELD OF THE INVENTION

The present invention relates generally to recombinant baculoviruses.

BACKGROUND OF THE INVENTION

5 Baculovirus infects insects and is non-pathogenic to humans, but can transduce a broad range of mammalian and avian cells. Thanks to the biosafety, large cloning capacity, low cytotoxicity and non-replication nature in the transduced cells as well as the ease of manipulation and production, baculovirus has gained explosive popularity as a gene delivery vector for a wide variety of applications such as antiviral therapy, cancer therapy, regenerative medicine and vaccine.

10 U.S. Patent No. 7,527,967 (or US 2005/0208066) discloses a recombinant baculovirus that displays a fusion heterologous polypeptide on the surface of the baculovirus for use in generating an antibody or an immune response against a heterologus protein or virus in a subject in need thereof. The fusion heterologous polypeptides therein is made by fusing a heterologus antigen with the carboxyl terminal amino acids from 227 to 529 of baculovirus GP64 protein (FIG. 2C). The
15 construct therein contains a substantial portion of the extracellular domain of GP64 including B12D5 binding site. When it is used in immunization, the extracellular domain of GP64 may elicit an immune response and produce unintended antibodies such as useless anti-GP64 B12D5 antibody (Zhou et al. *Virology* 2006; 352(2): 427–437). GP64 B12D5 antibody is a neutralization antibody against baculovirus itself instead of a foreign antigen of interest. In addition, baculovirus is slightly
20 immunogenic to porcine.

Taiwanese Patent No. I368656 discloses a method of using the signal peptide, transmembrane domain and the cytoplasmic transduction domain from GP64 to present an antigen. The construct therein contains only the transmembrane domain and cytoplasmic transduction domain without the extracellular domain of GP64 (FIG. 2D). It has less expression of foreign antigen and is sensitive to
25 inactivation reagents (Premanand et al. *PLoS ONE* 2013; 8(2): e55536).

Therefore, a heretofore unaddressed need exists in the art to address the aforementioned deficiencies and inadequacies, especially in connection with a baculovirus vector that is insensitive to inactivation reagent and has improved immunogenicity.

Spenger et al. (Eur. J. Biochem. 269, 4458–4467, 2002) disclose altering the surface properties of
30 baculovirus *Autographa californica* NPV by insertional mutagenesis of the envelope protein gp64.

SUMMARY OF THE INVENTION

In one aspect, the invention relates to a vector comprising a transgene encoding a signal peptide and a fusion protein, wherein the fusion protein comprises: (a) a heterologous antigen; and (b) a C-

terminal region of baculovirus envelope GP64 protein, comprising at least 100 amino acid residues in length and lacking a B12D5 binding epitope located within the central basic region of the GP64 protein, the B12D5 binding epitope comprising the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 3; wherein the C-terminal region of the baculovirus envelope GP64 protein is located within a range of amino acid residues from a.a. 292 to a.a. 512 of SEQ ID NO: 1 and with the C-terminus at a.a. 512; and further wherein the heterologous antigen is located between the signal peptide and the C-terminal region of the GP64 protein.

In one embodiment of the invention, the vector of the invention is a recombinant baculovirus.

In another aspect, the invention relates to a recombinant baculovirus displaying on its envelope a fusion protein, the fusion protein comprising: (i) a heterologous antigen; and (ii) a C-terminal region of baculovirus envelope GP64 protein, comprising at least 100 amino acid residues in length and lacking a B12D5 binding epitope located within the central basic region of the GP64 protein, the B12D5 binding epitope comprising the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 3, wherein the C-terminal region of the baculovirus envelope GP64 protein is located within a range of amino acid residues from a.a. 292 to a.a. 512 of SEQ ID NO: 1 and with the C-terminus at a.a. 512; and further wherein the heterologous antigen is located at the N-terminus of the fusion protein.

In one embodiment of the invention, the genome of the recombinant baculovirus comprises a transgene that encodes a signal peptide and the fusion protein of the invention, wherein the heterologous antigen is located between the signal peptide and the C-terminal region of the GP64 protein.

In another embodiment of the invention, the transgene is operably linked to a promoter.

In another embodiment of the invention, the promoter is polyhedrin.

In another embodiment of the invention, the C-terminal region of the GP64 protein has from 186 to 220 amino acids in length.

In another embodiment of the invention, the C-terminal region of the GP64 protein lacks the amino acid sequence of SEQ ID NO: 4.

In another embodiment of the invention, the C-terminal region of the GP64 protein comprises the amino acids from amino acid residue 293 to amino acid residue 512 of SEQ ID NO: 1.

In another embodiment of the invention, the C-terminal region of the GP64 protein comprises amino acids from amino acid residue 327 to amino acid residue 512 of SEQ ID NO: 1.

In another embodiment of the invention, the N-terminus of the C-terminal region of the GP64 protein is located between amino acid residue 292 and amino acid residue 328 of SEQ ID NO: 1.

In another embodiment of the invention, the signal peptide comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 5, 6, 7, 8, 9, 10, 11, and 12.

Further in another aspect, the invention relates to an insect cell or a cell transduced with the vector or the recombinant baculovirus of the invention.

5 In another embodiment of the invention, the antigen is at least one selected from the group consisting of a protein from a pathogen, a protein from a cancer cell, and an immune checkpoint protein.

The pathogen may be at least one selected from the group consisting of human papillomavirus, porcine reproductive and respiratory syndrome virus, human immunodeficiency virus-1, Dengue
10 virus, hepatitis C virus, hepatitis B virus, porcine circovirus 2, classical swine fever virus, foot-and-mouth disease virus, Newcastle disease virus, transmissible gastroenteritis virus, porcine epidemic diarrhea virus, influenza virus, pseudorabies virus, parvovirus, swine vesicular disease virus, poxvirus, rotavirus, Mycoplasma pneumonia, herpes virus, infectious bronchitis, infectious bursal disease virus. The cancer may be at least one selected from the group consisting of non-small cell
15 lung cancer, breast carcinoma, melanoma, lymphomas, colon carcinoma, hepatocellular carcinoma, and any combination thereof. The immune check point protein may be at least one selected from the group consisting of PD-1, PD-L1, PD-L2, and CTLA-4.

In another embodiment of the invention, the antigen is at least one selected from the group consisting of classical swine fever virus envelope glycoprotein E2, porcine epidemic diarrhea virus
20 S1 protein, programmed cell death protein 1, and a tumor-associated antigen.

Yet in another aspect, the invention relates to use of a vector or a recombinant baculovirus according to the invention in the manufacture of a medicament for eliciting an antigen-specific immunogenic response in a subject in need thereof. Alternatively, the invention relates to use of a vector or a recombinant baculovirus according to the invention for eliciting an antigen-specific
25 immunogenic response in a subject in need thereof. The accompanying drawings illustrate one or more embodiments of the invention and, together with the written description, serve to explain the principles of the invention. Wherever possible, the same reference numbers are used throughout the drawings to refer to the same or like elements of an embodiment.

BRIEF DESCRIPTION OF THE DRAWINGS

30 FIG. 1A is a schematic drawing illustrating a baculovirus vector platform design according to one embodiment of the invention.

FIG. 1B is a schematic drawing illustrating a foreign antigen or foreign genes not only can be anchored onto the virus envelope but also expressed in the membrane fraction of insect cells by a gBac surface display platform. The rectangle represents a virus.

FIG. 2A is a schematic drawing showing a full-length GP64 protein. GP64 minimum (GP64₃₂₇₋₄₈₂), GP64 transmembrane domain (TM) (GP64₄₈₃₋₅₀₅); cytoplasmic transduction domain (CTD) (GP64₅₀₆₋₅₁₂).

FIG. 2B is a schematic drawing showing a baculovirus vector according to one embodiment of the invention. SP: signal peptide; TM: transmembrane domain, CTD: cytoplasmic transduction domain.

FIG. 2C is a schematic drawing showing a baculovirus vector design disclosed in United States Patent No. 7527967.

FIG. 2D is a schematic drawing showing a baculovirus vector design disclosed in Taiwan Patent No. I368656.

FIG. 3 is a graph showing an immune response induced by baculovirus vectors in mice post vaccination (left panel) and neutralization serum (SN) titer measurement of E2-gBac subunit vaccine (right panel) in mice. Each ELISA value shown is an average value from 5 mice. The difference between the gBac and Bac groups is statistically significant, $P \leq 0.05$. The strain of baculovirus used was AcNPV. The term "SN" stands for neutralization serum. The term "E2-gBac" stands for "baculovirus vector CSFV E2-gBac" according to the vector design of FIG. 2B. The term "E2-gBac (inactivated)" means the baculovirus was inactivated before it was used for immunization. The term "E2-Bac" stands for "baculovirus vector CSFV E2-Bac" according to the vector design of FIG. 2D.

FIG. 4 is a graph showing an immune response induced by baculovirus vectors in pigs post vaccination. Each ELISA value shown is an average value from 3 pigs. The difference between the gBac and gp64 groups is statistically significant, $P \leq 0.05$. The term "E2-gp64" stands for "baculovirus vector CSFV E2-gp64" according to the vector design of FIG. 2C.

FIG. 5 is a graph showing antibody titers against porcine epidemic diarrhea virus (PEDV) in 3 specific pathogen-free (SPF) pigs post vaccination with the baculovirus vector PEDV S1-gBac. The 3 SPF pigs were labeled as number 73, 74 and 75, respectively. The term "IFA" stands for immunofluorescent antibody. The results indicated that the serum from the vaccinated pigs could be recognized by PED virus.

FIGs. 6A-B are photographs of western blot showing that the antigens E2 of CSFV E2-gBac (FIG. 6A) and S1 of PEDV S1-gBac (FIG. 6B) from the Sf9 cell samples were detected.

FIGs. 6C-D are photographs of western blot showing that the antigens of hPD-1-gBac in the samples were recognized and detected by anti-gp64 mAb (left panel) and human PD-1 antibody (right panel), respectively.

DETAILED DESCRIPTION OF THE INVENTION

5 The present invention is more particularly described in the following examples that are intended as illustrative only since numerous modifications and variations therein will be apparent to those skilled in the art. Various embodiments of the invention are now described in detail. Referring to the drawings, like numbers indicate like components throughout the views. As used in the description herein and throughout the claims that follow, the meaning of “a”, “an”, and “the”
10 includes plural reference unless the context clearly dictates otherwise. Also, as used in the description herein and throughout the claims that follow, the meaning of “in” includes “in” and “on” unless the context clearly dictates otherwise. Additionally, some terms used in this specification are more specifically defined below.

DEFINITIONS

15 The terms used in this specification generally have their ordinary meanings in the art, within the context of the invention, and in the specific context where each term is used. Certain terms that are used to describe the invention are discussed below, or elsewhere in the specification, to provide additional guidance to the practitioner regarding the description of the invention. For convenience, certain terms may be highlighted, for example using italics and/or quotation marks. The use of
20 highlighting has no influence on the scope and meaning of a term; the scope and meaning of a term is the same, in the same context, whether or not it is highlighted. It will be appreciated that same thing can be said in more than one way. Consequently, alternative language and synonyms may be used for any one or more of the terms discussed herein, nor is any special significance to be placed upon whether or not a term is elaborated or discussed herein. Synonyms for certain terms are
25 provided. A recital of one or more synonyms does not exclude the use of other synonyms.

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 to which this invention pertains. In the case of conflict, the present document, including definitions will control.

A vector is a vehicle used to transfer genetic material to a target cell. A viral vector is a virus
30 modified to deliver foreign genetic material into a cell.

The term "gene transduction", "transduce", or "transduction" is a process by which a foreign DNA is introduced into another cell via a viral vector.

A signal sequence or signal peptide (sometimes referred to as signal sequence, targeting signal, localization signal, localization sequence, transit peptide, leader sequence or leader peptide) is a short (5-30 amino acids long) peptide present at the N-terminus of the majority of newly synthesized proteins that are destined towards the secretory pathway. These proteins include those that reside
5 either inside certain organelles (the endoplasmic reticulum, golgi or endosomes), secreted from the cell, or inserted into most cellular membranes.

The term "B12D5" refers to a monoclonal antibody against gp64. B12D5 has a binding epitope of KKRPTWRHNV (SEQ ID NO: 3) at 277-287 of gp64, or HRVKKRPPTW (SEQ ID NO: 2) located within the central region from residues 271 to 292 (SEQ ID NO: 4) of gp64. *See* Zhou et al.
10 (2006) *Supra*; Wu et al (2012) "A pH-Sensitive Heparin-Binding Sequence from Baculovirus gp64 Protein Is Important for Binding to Mammalian Cells but Not to Sf9 Insect Cells" *Journal of Virology*, Vol. 86 (1) 484-491.

Programmed cell death protein 1, also known as PD-1 and CD279 (cluster of differentiation 279), is a protein that in humans is encoded by the *PDCD1* gene. PD-1 is a cell surface receptor that
15 belongs to the immunoglobulin superfamily and is expressed on T cells and pro-B cells. PD-1 binds two ligands, PD-L1 and PD-L2. PD-1, functioning as an immune checkpoint, plays an important role in down regulating the immune system by preventing the activation of T-cells, which in turn reduces autoimmunity and promotes self-tolerance. The inhibitory effect of PD-1 is accomplished through a dual mechanism of promoting apoptosis (programmed cell death) in antigen specific T-cells in
20 lymph nodes while simultaneously reducing apoptosis in regulatory T cells (suppressor T cells). A new class of drugs that block PD-1, the PD-1 inhibitors, activate the immune system to attack tumors and are therefore used with varying success to treat some types of cancer.

An antigen may be a pathogenic protein, polypeptide or peptide that is responsible for a disease caused by a pathogen, or is capable of inducing an immunological response in a host infected by the
25 pathogen, or tumor-associated antigen (TAA) which is a polypeptide specifically expressed in tumor cells. The antigen may be selected from a pathogen or cancer cells including, but not limited to, Human Papillomavirus (HPV), Porcine reproductive and respiratory syndrome virus (PRRSV), Human immunodeficiency virus-1(HIV-1), Dengue virus, Hepatitis C virus (HCV), Hepatitis B virus (HBV), Porcine Circovirus 2 (PCV2), Classical Swine Fever Virus (CSFV), Foot-and-mouth
30 disease virus (FMDV), Newcastle disease virus (NDV), Transmissible gastroenteritis virus (TGEV), Porcine epidemic diarrhea virus (PEDV), Influenza virus, Pseudorabies virus, Parvovirus, Pseudorabies virus, Swine vesicular disease virus (SVDV), Poxvirus, Rotavirus, Mycoplasma pneumonia, Herpes virus, infectious bronchitis, or infectious bursal disease virus, non-small cell lung

cancer, breast carcinoma, melanoma, lymphomas, colon carcinoma, hepatocellular carcinoma and any combination thereof. For example, HPV E7 protein (E7), HCV core protein (HCV core), HBV X protein (HBx) were selected as antigens for vaccine development. The antigen may be a fusion antigen from a fusion of two or more antigens selected from one or more pathogenic proteins. For example, a fusion antigen of PRRSV ORF6 and ORF5 fragments, or a fusion of antigens from PRRSV and PCV2 pathogens.

Alternatively, an antigen may be an inhibitory immune checkpoint protein such as PD-1, PD-L1, PD-L2, and CTLA-4, etc.

The terms “immune checkpoint protein” and “Immune checkpoint” are interchangeable. Immune checkpoints affect immune system functioning. Immune checkpoints can be stimulatory or inhibitory. Tumors can use these checkpoints to protect themselves from immune system attacks. Checkpoint therapy can block inhibitory checkpoints, restoring immune system function. One ligand-receptor interaction under investigation is the interaction between the transmembrane programmed cell death 1 protein (PDCD1, PD-1; also known as CD279) and its ligand, PD-1 ligand 1 (PD-L1, CD274). PD-L1 on the cell surface binds to PD1 on an immune cell surface, which inhibits immune cell activity. Among PD-L1 functions is a key regulatory role on T cell activities. It appears that (cancer-mediated) upregulation of PD-L1 on the cell surface may inhibit T cells that might otherwise attack. Antibodies that bind to either PD-1 or PD-L1 and therefore block the interaction may allow the T-cells to attack the tumor. Ipilimumab is the first checkpoint antibody approved by the FDA. It blocks inhibitory immune checkpoint CTLA-4.

The term “treating” or “treatment” refers to administration of an effective amount of the fusion protein to a subject in need thereof, who has cancer or infection, or a symptom or predisposition toward such a disease, with the purpose of cure, alleviate, relieve, remedy, ameliorate, or prevent the disease, the symptoms of it, or the predisposition towards it. Such a subject can be identified by a health care professional based on results from any suitable diagnostic method.

The term “an effective amount” refers to the amount of an active compound that is required to confer a therapeutic effect on the treated subject. Effective doses will vary, as recognized by those skilled in the art, depending on route of administration, excipient usage, and the possibility of co-usage with other therapeutic treatment.

EXAMPLES

Exemplary instruments, apparatus, methods and their related results according to the embodiments of the present invention are given below.

EXAMPLE 1

Construction of Vectors and Generation of Recombinant Baculoviruses, Virus-like Particles, and Proteins

FIG. 1A shows a gBac platform. A foreign gene (e.g., optimized classical swine fever virus (CSFV) E2 or porcine epidemic diarrhea virus (PEDV) spike genes) may be obtained by PCR
5 synthesis and then cloned into a gBac vector through restriction enzyme sites (e.g., *SacI*/*NotI*) using IN-FUSION® cloning kit (Clontech). The gBac vector is derived from baculovirus transfer vector pBACPAK8™ (GENBANK™ accession No. U02446). Using the gBac surface display platform of FIG. 1A, a foreign antigen or foreign genes can be anchored onto the virus envelope and also expressed in the membrane fraction of insect cells (FIG. 1B).

10 FIG. 2A shows a full-length GP64. The baculovirus GP64 envelope fusion protein (GP64 EFP) is the major envelope fusion glycoprotein in some, though not all, baculoviruses. It is found on the surface of both infected cells and budded virions as a homotrimer. Baculovirus enters its host cells by endocytosis followed by a low-pH-induced fusion of the viral envelope with the endosomal membrane, allowing viral entrance into the cell cytoplasm. This membrane fusion, and also the
15 efficient budding of virions from the infected cell, is dependent on GP64.

FIGs. 2B-D show comparisons of three vectors, gBac (or Reber-gBac), antigen-gp64 (or abbreviated as “gp64” disclosed in US 7527967), and Antigen-Bac (or abbreviated as “Bac” disclosed in TW I368656). The gBac vector was constructed using the signal peptide (SP) and a C-terminal region of GP64 from amino acids 327 to 513. The gp64 vector was constructed using the
20 SP and a C-terminal region of GP64 from amino acids 227 to 513. The Bac vector comprises the insect signal peptide (SP), the TM (transmembrane domain) and CTD (cytoplasmic transduction domain) of GP64 and does not comprise the GP64 extracellular domain amino acids. The symbol triangle before the “SP” represents the polyhedrin promoter, which is located -1 site of the insert gene's start codon.

25 A DNA fragment encoding the SP was generated and amplified by PCR with forward and reverse primers 5'-GAGCTCATGGTAAGC-3' (SEQ ID NO: 19) and 5'-AGGCAGAATGCG-3' (SEQ ID NO: 20), respectively. The C-terminal portion of the gp64 gene (encoding GP64 from aa 327 to aa 513) was generated and amplified by PCR using the forward and reverse primers 5'-GCGTGTCTGCTCA-3' (SEQ ID NO: 21) and 5'-TTAATATTGTCTA-3' (SEQ ID NO: 22),
30 respectively. The C-terminal portion of the gp64 gene (encoding GP64 from aa 227 to aa 513) was generated and amplified by PCR using the forward and reverse primers 5'-ATCAACAAGCTAA-3' (SEQ ID NO: 23) and 5'-TTAATATTGTCTA-3' (SEQ ID NO: 24), respectively. The above foreign genes were reselectively cloned into the pBACPAK8™ transfer vector.

Using the gBac platform of 2B (or FIG. 1A), we generated two baculovirus vectors: the baculovirus vector CSFV E2-gBac and the baculovirus vector PEDV S1-gBac (FIG. 2B design). The former transduces a gene encoding classical swine fever virus (CSFV) envelope glycoprotein E2, and the latter transduces a gene encoding porcine epidemic diarrhea virus S1 Protein.

For a parallel comparison, we have also used the vectors of FIGs. 2C and 2D and generated two separate baculovirus vectors: the baculovirus vector CSFV E2-gp64 (FIG. 2C design), and the baculovirus vector CSFV E2-Bac (FIG. 2D design) for transducing a foreign gene encoding the antigen CSFV E2.

The fusion genes in the gBac, gp64, and Bac vectors (FIGs. 2B-D designs, respectively) were sequenced to confirm their identities. Recombinant baculovirus viruses containing the recombinant GP64 gene without the transfer vector backbone were generated by homologous recombination. Methods for this construction and recombination are well known in the art. These recombinant viruses were used to prepare antigens. The modified GP64 gene was inserted into a baculovirus transfer vector under the polyhedron promoter by PCR to form the gBac vector. Because the gBac vector has two homologous recombination sites, the original polyhedron locus of wild-type baculovirus is replaced by gBac sequence when co-transfection with gBac plasmid and wild-type baculovirus.

EXAMPLE 2

Protein expression and purification of antigens

Sf9 cells were used for propagation and infection of recombinant baculovirus. Baculovirus was propagated in *Spodoptera frugiperda* Sf-9 cell lines and grown at 26°C in a serum-free medium. The recombinant baculoviruses were produced by infecting Sf-9 cells at an MOI of 5-10 and harvested 3 days after infection. To produce baculovirus on a large scale, the cells may be cultured and infected in bioreactors. The baculovirus fraction were isolated from the culture supernatant of the infected cell. The baculovirus particles were collected by Tangential Flow Filtration (TFF) using a suitable protein cut-off membrane and 0.45 µm sterile membrane.

To test the production of the antigens, the partially purified baculoviruses or infected cell lysates were subjected to 8-10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The foreign antigens were detected by mouse anti-gp64 mAb (available from commercial sources and used at 1:5,000 dilution) as the primary antibody. The secondary antibodies were goat anti-rabbit or goat anti-mouse mAb conjugated with alkaline phosphatase (1:5,000 dilution). The protein bands were visualized by ECL PLUS™ Western Blotting Detection Reagents (GE Healthcare). In the baculovirus surface display system, the foreign antigens

can be firstly expressed on insect cell membrane, then the budding baculovirus take some of cell membrane to form its envelope. As the result, baculovirus display antigens can be harvested from infected cell lysate (membrane fraction) and virus surface.

EXAMPLE 3

5 Expression of the antigens by recombinant viruses

Before immunization with gBac vaccines, to confirm whether the fusion protein CSFV E2-gBac or PEDV S1-gBac were successfully displayed on the baculovirus envelope, recombinant baculoviruses were collected by centrifugation and TFF. The recombinant baculoviruses were re-suspended in PBS at different concentrations of p.f.u./ μ l. Incorporation of recombinant proteins into
10 the baculovirus particles were analyzed by Western blotting using anti-gp64 mAb (eBioscience). The fusion protein CSFV E2-gBac was detected at a molecular weight of about 80 kDa and the fusion protein PEDV S1-gBac was detected at a molecular weight of 130 kDa.

EXAMPLE 4

Vaccine preparation and immunization

15 The recombinant baculoviruses displaying antigens were mixed with water-in-oil-in-water (w/o/w) adjuvant (MONTANIDE ISA 206 VG, SEPPIC.) for vaccine preparation. Female BALB/c mice of 4-week old, purchased from National Experimental Animal Center, Taiwan, R.O.C., were divided into four groups with 5 mice per group. Mice were immunized subcutaneously twice on day 0 and day-14 (FIG. 3) with 200 μ l of a solution containing with 10^7 p.f.u. recombinant baculovirus.
20 The virus of E2-gBac, E2-gp64, and E2-Bac, (FIGs. 2B-D), containing CSFV envelope glycoprotein E2, were inactivated and formulated as vaccines. As negative controls, mice were injected with wild type baculovirus (10^7 p.f.u.) or PBS. The blood samples were collected from the caudal vein at week 6. Binary ethylenimine (BEI) was used to inactivate baculoviruses. It was prepared by dissolving the 0.1 M 2-bromoethylamine hydrobromide (BEA) in 0.2 N NaOH at 37°C for 1 h. To inactivate the
25 baculovirus, the viral medium was added 4 mM BEI and virus incubated for 16 h at 37°C. After inactivation, sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$) was added to the medium at a final concentration of 10 times the final BEI concentration to stop the inactivation.

EXAMPLE 5

Immunogenicity of antigens produced by recombinant viruses

30 After immunization, the sera of mice were analyzed for the presence of anti-CSFV E2 antibody using the IDEXX CSFV Ab Test Kit (IDEXX) to detect classical swine fever virus (CSFV) antibodies. The degree of CSFV E2-specific antibodies in the serum was calculated as positive when the blocking percentage was above 40% (FIG. 3). The results indicate that the baculovirus vector

displayed the fusion protein CSFV E2-Bac. FIG. 3 show that the baculovirus vector CSFV E2-gBac, whether the baculovirus was inactivated or not, induced a much higher anti-CSFV E2 antibody titer than the baculovirus vector CSFV E2-Bac in mice.

We have also compared the effects of the three baculovirus vectors in inducing immunogenic responses in pigs. Three SPF (specific pathogen free) pigs were immunized twice (6-week-old and 9-week-old) via intramuscular route with 10^8 pfu of recombinant baculovirus vectors E2-gBac, E2-gp64, E2-Bac, and PBS, respectively. After immunization, the sera of piglets were analyzed for the presence of CSFV E2 antibody using IDEXX CSFV Ab Test Kit (IDEXX). The degree of CSFV E2 specific neutralization antibody in the serum was calculated as positive and efficient when the blocking percentage was above 43%.

As shown in FIG. 4, pigs immunized with E2-gBac were able to survive the CSFV challenge. It indicates that competent neutralization antibody had been induced in the pigs (SN titer ≥ 16 , IDEXX blocking ratio $\geq 43\%$). This proves that the gBac platform is a vaccine platform. FIG. 4 shows that the baculovirus vector CSFV E2-gBac induced a much higher anti-CSFV E2 antibody titer than the baculovirus vectors CSFV E2-Bac and CSFV E2-gp64 in pigs. The results indicate that the gBac surface display platform of the invention can induce a stronger immunity than the prior art baculovirus vectors.

We have also generated baculovirus vector for transducing porcine epidemic diarrhea virus S1 protein (PEDV S1) into cells for vaccination applications. We tested its effects in inducing an immunogenic response. Briefly, three 9-week-old SPF pigs (labeled as number 73, 74 and 75, respectively) were each immunized twice via an intramuscular route with 10^8 pfu of recombinant baculovirus vector PEDV S1-gBac or PBS. After immunization, the sera of pigs (from 1 to 4 weeks post vaccination) were analyzed for the presence of anti-PEDV antibodies using an ELISA assay of PED virus. FIG. 5 shows that the baculovirus vector PEDV S1-gBac induced antibody titers against porcine epidemic diarrhea virus in all 3 pigs.

FIGS. 6A-B are photographs of Western blots showing that the antigens E2 of CSFV E2-gBac (FIG. 6A) and S1 of PEDV S1-gBac (FIG. 6B) from the Sf9 cell samples were detected. The cell lysates and collected virus were loaded by different cell number and virus titer. We have also constructed the baculovirus vector human programmed cell death protein 1 (hPD-1gBac)-gBac (hPD-1gBac). To test the production of the antigen, the recombinant baculoviruses or infected cell lysates were subjected to 8-10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The foreign antigens were detected by a mouse anti-gp64 mAb (FIG. 6C) and anti-PDCD-1 antibody (FIG. 6D) (Santa Cruz Biotechnology, Santa

Cruz, CA) as the primary antibodies. The protein bands were visualized by ECL PLUS™ Western Blotting Detection Reagents (GE Healthcare). FIG. 6D shows that the baculovirus displayed human PD-1 antigen on the envelope, and the displayed PD-1 antigen could be recognized by commercial anti-PDCD-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

- 5 In summary, the vector of the invention is insensitive to inactivation reagents (FIG. 3) and exhibits higher immunogenicity (FIG. 4). Table 1 shows peptide sequences and SEQ ID NOs.

Table 1

Protein or peptide	Amino acid sequence* (SEQ ID NO:)	a.a. length
Full length GP64	<u>MVSAIVLYVLLAAAAHSAFA</u> AHCNAQMKTGPYKIKNLDITPPKET LQKDVEITIVETDYNENVIIGYKGYQAYAYNGGSLDPNTRVEE TMKTLNVGKEDLLMWSIRQQCEVGEELIDRWGSDSDDCFRDNE GRGQWVKGKELVKRQNNNHFAHHTCNKSWRCGISTSKMYSRL ECQDDTDECQVYILDAEGNPINVTVDTVLHRDGVSMILKQKSTF TTRQIKAACLLIKDDKNNPESVTREHCLIDNDIYDLSKNTWNCK FNRCIKRKVEHRVKKRPPTWRHNVRAKYTEGDTATKGDLMHIQ EELMYENDLLKMNIELMHAH <u>INKLNNMLHDLIVSVAKVDERLI</u> <u>GNLMNNSVSSTFLSDDTFLMPCTNPPAHTSNCYNNSIYKEGRW</u> <u>VANTDSSQCIDFSNYKELAIDDDVEFWIPTIGNTTYHDSWKDAS</u> <u>GWSFIAQQKSNLITTMENKTFGGVGTSLSDITSMAEGELAAKLT</u> <u>SFMFGHVVN FVILIVILFLYCMIRNRNRQY</u> (SEQ ID NO: 1)	512
B12D5 binding epitope	HRVKKRPPTW (SEQ ID NO: 2; 274-283 of GP64).	
B12D5 binding epitope	KKRPPTWRHNV (277-287 of gp64; SEQ ID NO: 3)	
Gp64 central basic region	KVEHRVKKRPPTWRHNVRAKYT (271-292 of gp64; SEQ ID NO: 4)	
GP64 signal peptide (SP)	MVSAIVLYVLLAAAAHSAFA (GP64 ₁₋₂₀ ; SEQ ID NO: 5)	20
30K protein <i>Bombyx mori</i>	MRLTLFAFVLAVCALASNA (SEQ ID NO: 6)	
SP1	MRVLVLLACLAAASA	

<i>Bombyx mori</i>	(SEQ ID NO: 7)	
SP2 <i>Bombyx mori</i>	MKSVLILAGLVAVALSSAVPKP (SEQ ID NO: 8)	
Bombyxin A-4	MKILLAIALMLSTVMWVST (SEQ ID NO: 9)	
Vitellogenin	MKLFVLAIIIAVSS (SEQ ID NO: 10)	
Chitinase precursor	MRAIFATLAVLASCAALVQS (SEQ ID NO: 11)	
Adipokinetic hormone	MYKLTVFLMFI AF VIIAGAQS MASLTRQDLA (SEQ ID NO: 12)	
CSFV E2 (Classical swine fever virus (CSFV) envelope glycoprotein E2) <i>CSFV 96TD</i>	MLRGQVVQGIIWLLVTGAQGRLSCKEDHRYAISSTNEIGPLGA EGLTTTWKEYNHGLQLDDGTVRAICIAGSFKVTALNVVSRRYL ASLHKRALPTSVTFELLFDGTSPAIEEMGDDFGFGLCPFDTPVV KGKYNTTLLNGSAFYLVCPIGWTGVIECTAVSPTTLRTEVVKTF KREKPPHRVDCVTTIVEKEDLFYCKLGGNWTCVKGNPVTYTG GQVRQCRWCGFDFKEPDGLPHYPIGKCILTNETGYRVVDSPDCN RDGVVISTEGEHECLIGNTTVKVHALDGR LAPMPCRPK EIVSSA GPVRKTSCTFNYTKTLRNKY YEP RDSYFQQYMLKGEYQYWFDL DVTDHHTDYFAEF (SEQ ID NO: 13)	363
PEDV S1 (Porcine Epidemic Diarrhea Virus S1 Protein) <i>PEDV</i> <i>USA/Iowa/1898</i> <i>4/2013</i>	CSANTNFRFFSKFNVQAPAVVVLGGYLP IGENQGVNSTWYCA GQHPTASGVHGFVSHIRGGHGFEIGISQEPDPSGYQLYLHKAT NGNTNATARLRICQFPSIKTLGPTANNDVTTGRNCLFNKAIPAH MSEHSVVGITWDNDRVTVFSDKIYYFYFKNDWSRVATKCYNSG GCAMQYVYEPTY YMLNVT SAGEDGISYQPCTANCIGYAANVFA TEPNGHIPEGFSFNNWFLLSNDSTLVHGKVVS NQPLL VNCLLAIP KIYGLGQFFSFNQ TIDGVCNGAAVQRAPEALRFNINDTSVILAEG SIVLHTALGTNFSFVCSNSSNPHLATFAIPLGATQVPYYCFFKVD TYNSTVYKFLAVLPPTVREIVITKYGDVYVNGFGYLHLGLLDAV TINFTGHGTDDDVS GFWTIASTNFVDALIEVQGTAIQRILYCDDP VSQKCSQVAFDLDDGFYPISSRNLLSHEQPISFVTLPSFNDHSFV NITVSASFGGHSGANLIASDTTINGFSSFCVDTRQFTISLFYNVTN SYGYVSKSQDSNCPFTLQSVNDYLSFSKFCVSTSLLASACTIDLF	708

	GYPEFGSGVKFTSLYFQFTKGELITGTPKPLEGVTDVVSFMTLDVC TKYTIYGFKGEGIIITLTNSSFLAGVYYTSDSGQLLAFKNVTSGAV YSVTPCSFSEQAAYVDDDIVGVISSLSSSTFNSTRELPG (SEQ ID NO: 14)	
Human PD-1 (Programmed cell death protein 1)	QIPQAPWPVVWAVLQLGWRPGWFLDSPDRPWNPPFFPALLVV TEGDNATFTCSFSNTSESVLWYRMSPSNQTDKLAAPEDRSQ PGQDCRFRVTQLPNGRDFHMSVVRARRNDSGYLCGAISLAPK AQIKESLRAELRVTERRAEVPTAHPSPSPRPAGQFQTDIY (SEQ ID NO: 15)	170
E2-gBac protein (SP-E2-GP64 mini-TM/CTD)	<u>MVSAIVLYVLLAAAAHSAF</u> AMLRGQVVQGIWLLLVGTGAQGRLSC KEDHRYAISSTNEIGPLGAEGLTWKEYNHGLQLDDGTVRAICI AGSFKVTALNVVSRRYLASLHKRALPTSVTFELFDGTSPAIEEM GDDFGFGLCPFDTPPVVKGYNTLLNGSAFYLVCPIGWTGVIE CTAVSPTTLRTEVVKTFKREKPFPHRVDCVTTIVEKEDLFYCKLG GNWTCVKGNPVITYTGGQVRQCRWCGDFKEPDGLPHYPIGKCI LTNETGYRVVDSPDCNRDGVVISTEGEHECLIGNTTVKVHALDG RLAPMPCRPKIEVSSAGPVRKTSCTFNKYTKLRNKYYEPRDSYF QQYMLKGEYQYWFDLDVTDHHTDYFAEFINKLNNMLHDLIVS <u>VAKVDERLIGNLMNNSVSSTFLSDDTFLMPCTNPPAHTSNCYN</u> <u>NSIYKEGRWVANTDSSQCIDFSNYKELAIDDDVEFWIPTIGNTTY</u> <u>HDSWKDASGWSFIAQOKSNLITTMNTKFGGVGTSLSDITSMAE</u> <u>GELAAKLTSFMFGHVVNFIILIVILFLYCMIRNRNRQY</u> (SEQ ID NO: 16)	569
S1-gBac protein (SP-S1-GP64 mini-TM/CTD)	<u>MVSAIVLYVLLAAAAHSAF</u> ACSANTNFRFFSKFNVQAPAVVVLGG YLPIGENQGVNSTWYCAGQHPTASGVHGFVSHIRGGHGFEIGIS QEPFDPSGYQLYLHKATNGNTNATARLRICQFPSIKTLGPTANN DVTTGRNCLFNKAIPAHMSEHSVVGITWDNDRVTVFSDKIYYFY FKNDWSRVATKCYNSGGCAMQYVYEPTYMLNVTSAAGEDGIS YQPCTANCIGYAANVFATEPNNGHIPEGFSFNNWFLLSNDSTLVH GKVVSNQPLLVNCLLAIPKIYGLGQFFSFNQITIDGVCNGAAVQR APEALRFNINDTSVILAEGSIVLHTALGTNFSFVCSNSSNPHLATF AIPLGATQVPYYCFFKVDTYNSTVYKFLAVLPPTVREIVITKYGD VYVNGFGYLHLGLLDAVTINFTGHGTDDDVSFGFTIASTNFVD	914

	ALIEVQGTAIQRILYCDDPVSQLKCSQVAFDLDDGFYPISSRNLLS HEQPISFVTLPSFNDHSFVNITVSASFSGHSGANLIASDTTNGFSS FCVDTRQFTISLFYNVTNSYGYVSKSQDSNCPFTLQSVNDYLSFS KFCVSTSLLASACTIDLFGYPEFGSGVKFTSLYFQFTKGELITGTP KPLEGVTDVSFMTLDVCTKYTIYGFKGEGIITLTNSSFLAGVYYT SDSGQLLAFKNVTSGAVYSVTPCSFSEQAAYVDDDIVGVISLSS STFNSTRELPGIN <u>KLNNMLHDLIVSVAKVDERLIGNLMNNSVSST</u> <u>FLSDDTFLLMPCTNPPAHTSNCYNNSIYKEGRWVANTDSSQCID</u> <u>FSNYKELAIDDDDVEFWIPTIGNTTYHDSWKDASGWSFIAQOKSN</u> <u>LITTMENKFGGVGTSLSDITSMAEGELAAKLTSFMFGHVNVFV</u> IILIVILFLYCMIRNRNRQY (SEQ ID NO: 17)	
hPD1-gBac protein (SP-hPD1-GP64 mini-TM/CTD)	<u>MVSAIVLYVLLAAAAHSAFAQ</u> IPQAPWPVWAVLQLGWRPGWFL DSPDRPWNPPTEFFPALLVVTEGDNATFTCSFSNTSESFVLNWYR MSPSNQTDKLAAPEDRSQPGQDCRFRVTQLPNGRDFHMSVVR ARRNDSGTYLCGAISLAPKAQIKESLRAELRVTERRAEVPTAHPS PSPRPAGQFQTDIYIN <u>KLNNMLHDLIVSVAKVDERLIGNLMNNS</u> <u>VSSTFLSDDTFLLMPCTNPPAHTSNCYNNSIYKEGRWVANTDSS</u> <u>QCIDFSNYKELAIDDDDVEFWIPTIGNTTYHDSWKDASGWSFIAQ</u> <u>OKSNLITTMENKFGGVGTSLSDITSMAEGELAAKLTSFMFGHV</u> VNFVILIVILFLYCMIRNRNRQY (SEQ ID NO: 18)	376

*Full length GP64: The GP64 Signal peptide (SP) (GP64₁₋₂₀) is underlined and in italics. The GP64 minimum (GP64₃₂₇₋₄₈₂) is underlined without italics. The GP64 transmembrane domain (TM) (GP64₄₈₃₋₅₀₅) is in bold only. The GP64 cytoplasmic transduction domain (CTD) (GP64₅₀₆₋₅₁₂) is in italics only.

E2-gBac protein (SP-E2-GP64 mini-TM/CTD): The GP64 Signal peptide (SP) (GP64₁₋₂₀) is underlined and in italics. The GP64 minimum (GP64₃₂₇₋₄₈₂) is underlined without italics. The GP64 transmembrane domain (TM) (GP64₄₈₃₋₅₀₅) is in bold only. The GP64 cytoplasmic transduction domain (CTD) (GP64₅₀₆₋₅₁₂) is in italics only.

S1-gBac protein (SP-S1-GP64 mini-TM/CTD): The GP64 Signal peptide (SP) (GP64₁₋₂₀) is underlined and in italics. The GP64 minimum (GP64₃₂₇₋₄₈₂) is underlined without italics. The GP64 transmembrane domain (TM) (GP64₄₈₃₋₅₀₅) is in bold only. The GP64 cytoplasmic transduction domain (CTD) (GP64₅₀₆₋₅₁₂) is in italics only.

hPD1-gBac protein (SP-hPD1-GP64 mini-TM/CTD): The GP64 Signal peptide (SP) (GP64₁₋₂₀) is underlined and in italics. The GP64 minimum (GP64₃₂₇₋₄₈₂) is underlined without italics. The GP64 transmembrane domain (TM) (**GP64₄₈₃₋₅₀₅**) is in bold only. The GP64 cytoplasmic transduction domain (CTD) (*GP64₅₀₆₋₅₁₂*) is in italics only.

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CLAIMS

What is claimed is:

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1. A recombinant baculovirus displaying on its envelope a fusion protein, the fusion protein comprising:
 - (i) a heterologous antigen; and
 - (ii) a C-terminal region of baculovirus envelope GP64 protein, comprising at least 100 amino acid residues in length and lacking a B12D5 binding epitope located within the central basic region of the GP64 protein, the B12D5 binding epitope comprising the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 3,wherein the C-terminal region of the baculovirus envelope GP64 protein is located within a range of amino acid residues from a.a. 292 to a.a. 512 of SEQ ID NO: 1 and with the C-terminus at a.a. 512; and
further wherein the heterologous antigen is located at the N-terminus of the fusion protein.

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2. The recombinant baculovirus of claim 1, the genome of which comprises a transgene that encodes a signal peptide and the fusion protein,
wherein the heterologous antigen is located between the signal peptide and the C-terminal region of the GP64 protein.

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3. A vector comprising a transgene encoding a signal peptide and a fusion protein, wherein the fusion protein comprises:
 - (a) a heterologous antigen; and
 - (b) a C-terminal region of baculovirus envelope GP64 protein, comprising at least 100 amino acid residues in length and lacking a B12D5 binding epitope located within the central basic region of the GP64 protein, the B12D5 binding epitope comprising the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 3;wherein the C-terminal region of the baculovirus envelope GP64 protein is located within a range of amino acid residues from a.a. 292 to a.a. 512 of SEQ ID NO: 1 and with the C-terminus at a.a. 512; and

further wherein the heterologous antigen is located between the signal peptide and the C-terminal region of the GP64 protein.

4. The vector of claim 3, which is a recombinant baculovirus.

5. The vector of claim 3, wherein the transgene is operably linked to a promoter.

6. The recombinant baculovirus of claim 1, wherein the C-terminal region of the GP64 protein lacks the amino acid sequence of SEQ ID NO: 4.

7. The recombinant baculovirus of claim 1, wherein the C-terminal region of the GP64 protein comprises amino acids from amino acid residue 327 to amino acid residue 512 of SEQ ID NO: 1.

8. The recombinant baculovirus of claim 1, wherein the N-terminus of the C-terminal region of the GP64 protein is located between amino acid residue 292 and amino acid residue 328 of SEQ ID NO: 1.

9. The recombinant baculovirus of claim 2, wherein the signal peptide comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 5, 6, 7, 8, 9, 10, 11, and 12.

10. An insect cell transduced with the recombinant baculovirus of claim 1.

11. The recombinant baculovirus of claim 1, wherein the antigen is at least one selected from the group consisting of a protein from a pathogen a protein from a cancer cell, and an immune checkpoint protein.

12. The recombinant baculovirus of claim 11, wherein:

(i) the pathogen is at least one selected from the group consisting of human papillomavirus, porcine reproductive and respiratory syndrome virus, human immunodeficiency virus-1, Dengue virus, hepatitis C virus, hepatitis B virus, porcine circovirus 2, classical swine fever virus, foot-and-mouth disease virus, Newcastle disease virus, transmissible gastroenteritis virus, porcine epidemic diarrhea virus, influenza virus, pseudorabies virus, parvovirus, swine

vesicular disease virus, poxvirus, rotavirus, Mycoplasma pneumonia, herpes virus, infectious bronchitis, and infectious bursal disease virus;

(ii) the cancer cell is at least one selected from the group consisting of non-small cell lung cancer cells, breast carcinoma cells, melanoma cells, lymphoma cells, colon carcinoma cells, and hepatocellular carcinoma cells; and

(iii) the immune check point protein is at least one selected from the group consisting of PD-1, PD-L1, PD-L2, and CTLA-4.

13. The recombinant baculovirus of claim 1, wherein the antigen is at least one selected from the group consisting of classical swine fever virus envelope glycoprotein E2, porcine epidemic diarrhea virus S1 protein, programmed cell death protein 1, and a tumor-associated antigen.

14. The vector of claim 3, wherein the antigen is at least one selected from the group consisting of a pathogen protein, a cancer cell protein, and an immune checkpoint protein.

15. Use of the vector of claim 3 in the manufacture of a medicament for eliciting an antigen-specific immunogenic response in a subject in need thereof.

16. Use of the vector of claim 3 for eliciting an antigen-specific immunogenic response in a subject in need thereof.

17. Use of the recombinant baculovirus of claim 1 in the manufacture of a medicament for eliciting an antigen-specific immunogenic response in a subject in need thereof.

18. Use of the recombinant baculovirus of claim 1 for eliciting an antigen-specific immunogenic response in a subject in need thereof.

19. The vector of claim 3, wherein the C-terminal region of the GP64 protein-comprises the amino acids from amino acid residue 327 to amino acid residue 512 of SEQ ID NO: 1.

20. The vector of claim 3, wherein the C-terminal region of the GP64 protein-comprises the amino acids from amino acid residue 293 to amino acid residue 512 of SEQ ID NO: 1.

21. The recombinant baculovirus of claim 1, wherein the C-terminal region of the GP64 protein comprises the amino acids from amino acid residue 293 to amino acid residue 512 of SEQ ID NO: 1.

5 22. The recombinant baculovirus of claim 2, wherein the transgene is operably linked to a promoter.

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FIG. 1A

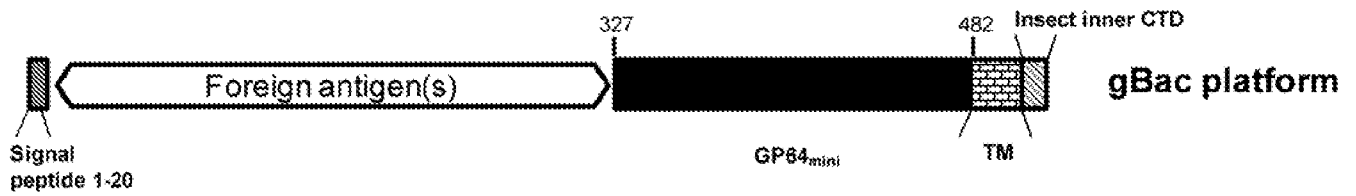


FIG. 1B

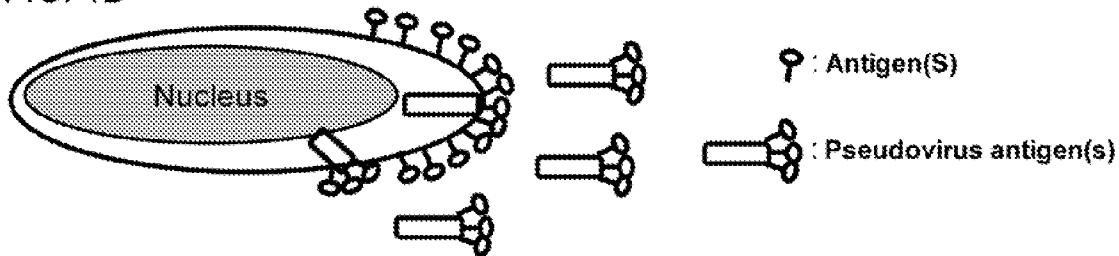
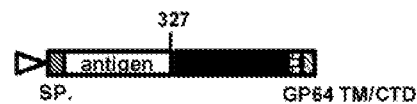


FIG. 2A



FIG. 2B

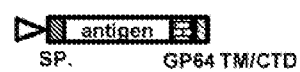


Reber-gBac

FIG. 2C

Antigen-gp64
(US7,527,967 B2)

FIG. 2D

Antigen-Bac
(TWI368656)

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FIG. 3

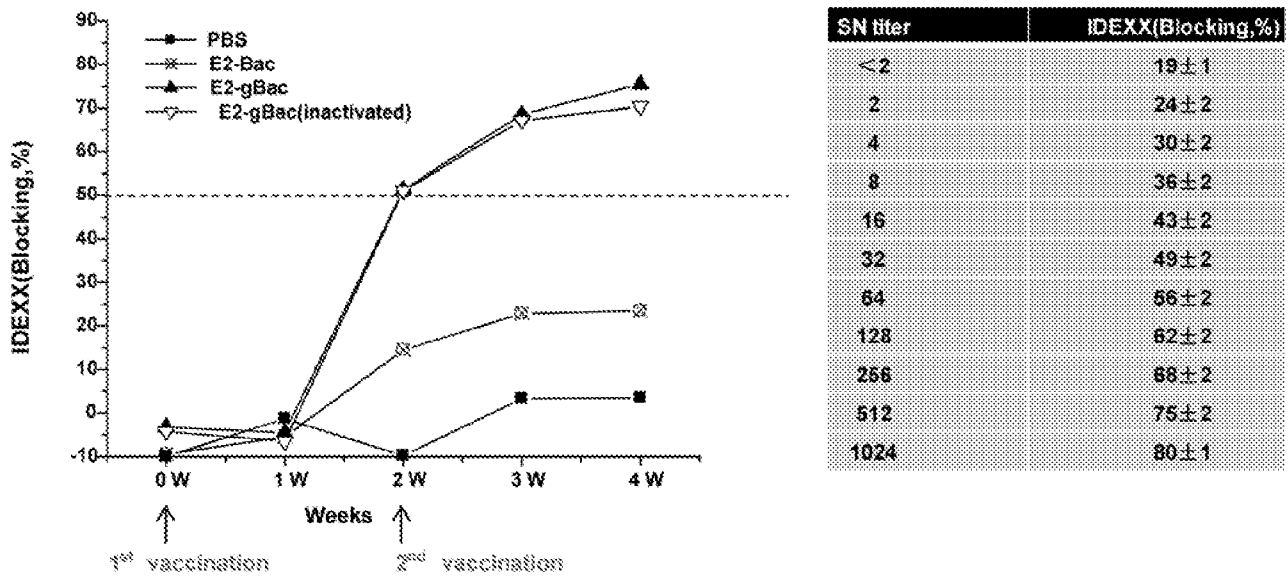


FIG. 4

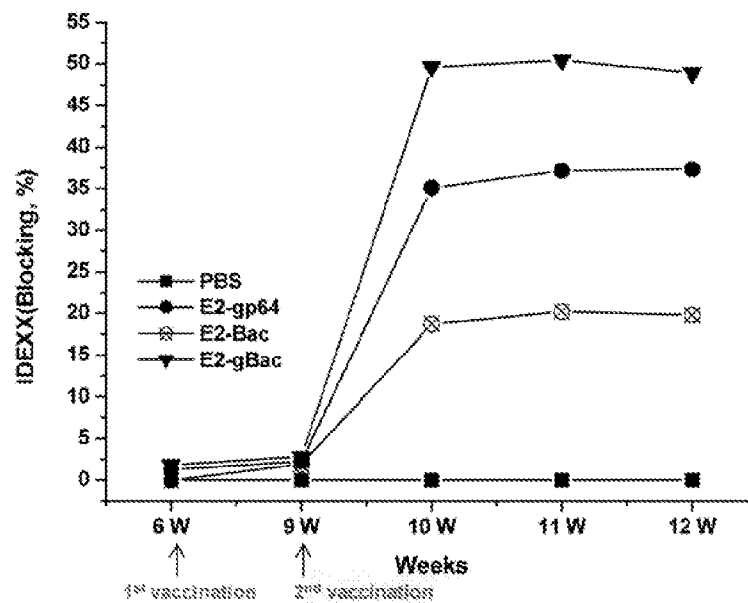


FIG. 5

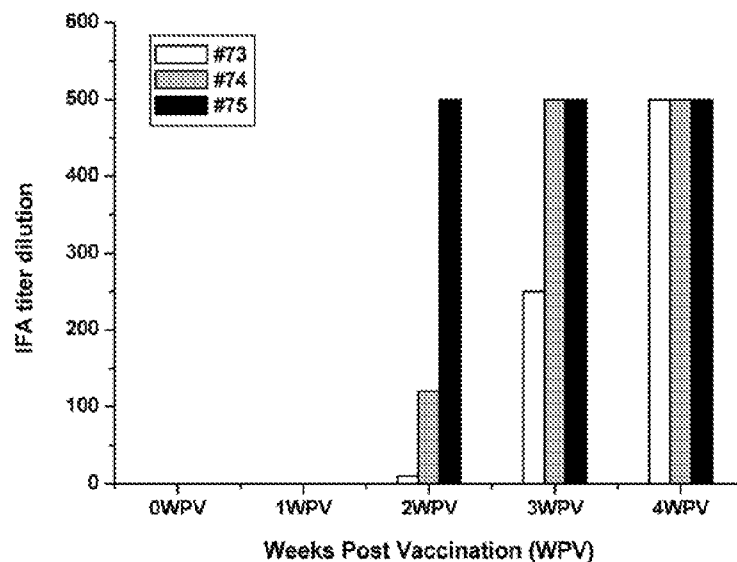


FIG. 6A

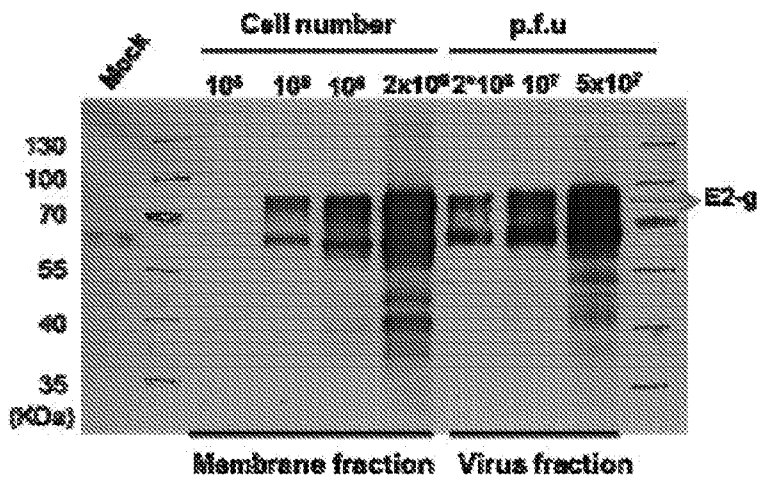


FIG. 6B

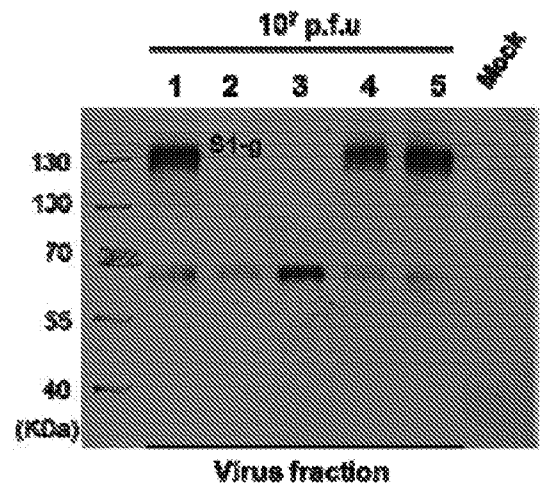


FIG. 6C

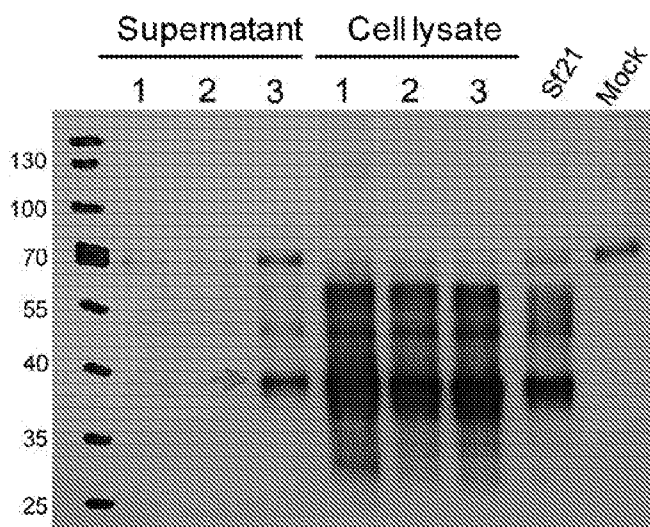
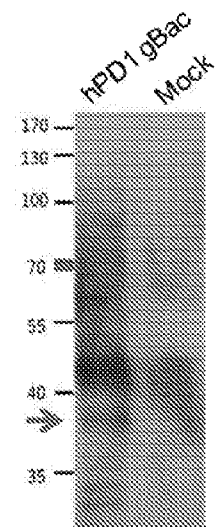
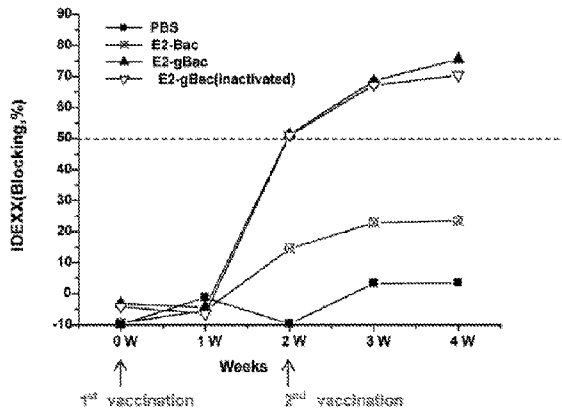


FIG. 6D





SN titer	IDXX(Blocking, %)
<2	19 ± 1
2	24 ± 2
4	30 ± 2
8	36 ± 2
16	43 ± 2
32	49 ± 2
64	56 ± 2
128	62 ± 2
256	68 ± 2
512	75 ± 2
1024	80 ± 1