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(54) Title: VIRUS LIKE PARTICLE COMPRISING MODIFIED ENVELOPE PROTEIN E3

(57) Abstract: A virus like particle comprising a viral structural protein which comprises modified envelope protein E3. The viral structural protein may be that derived from or alphavirus or flavivirus. Especially, the viral structural protein may be derived from Chikungunya virus or Venezuelan equine encephalitis virus.

Description

Title of Invention: VIRUS LIKE PARTICLE COMPRISING MODIFIED ENVELOPE PROTEIN E3

Technical Field

[0001] The present application relates to a virus like particle comprising a modified envelope protein E3, and use thereof.

Background Art

[0002] Virus-like particles (VLPs) are multiprotein structures that mimic the organization and conformation of authentic native viruses but lack the viral genome, potentially yielding safer and cheaper vaccine candidates. A handful of prophylactic VLP-based vaccines is currently commercialized worldwide: GlaxoSmithKline's Engerix^{R} (hepatitis B virus) and Cervarix^{R} (human papillomavirus), and Merck and Co., Inc.'s Recombivax HB^{R} (hepatitis B virus) and Gardasil^{R} (human papillomavirus) are some examples. Other VLP-based vaccine candidates are in clinical trials or undergoing pre-clinical evaluation, such as, influenza virus, parvovirus, Norwalk and various chimeric VLPs. Many others are still restricted to small-scale fundamental research, despite their success in preclinical tests. The implications of large-scale VLP production are discussed in the context of process control, monitorization and optimization. The main up- and down-stream technical challenges are identified and discussed accordingly. Successful VLP-based vaccine blockbusters are briefly presented concomitantly with the latest results from clinical trials and the recent developments in chimeric VLP-based technology for either therapeutic or prophylactic vaccination.

[0003] Up to now, VLP-based vaccines have been produced for more than 30 different viruses that infect human and other animals. The examples include AAV (Adeno-associated virus), H5N3 (Avian influenza), BFDV (Budgerigar fledgling disease virus), BTV (Bluetongue virus), Ebola, Enterovirus 71, GHPV (Goose hem-orrhagic polyoma virus), HBV (Hepatitis B virus), HCV (Hepatitis C virus), HDV (Hepatitis δ virus), HEV (Hepatitis E virus), HIV, HPV (Human papillomavirus), IBDV (Infectious bursal disease virus), Influenza A, Influenza A H1N1, Influenza A H3N2, JC polymavirus, Margurg, MS2, IPCV (Indian peanut clump virus), NDV (Newcastle disease virus), No (Norovirus) Nv (Norwalk virus), PhMV (Physalis mottle virus), Polymavirus, PPV (Porcine parvovirus), RHDV (Rabbit hemorrhagic disease virus), Rotavirus, SARS, SIV (Simian immunodeficiency virus), SV40 (Simian virus 40), SVDV (Swine vesicular disease virus) and so on. (Expert Rev. Vaccines 9(10), 1149-1176, 2010).

[0004] To quickly generate large quantity of VLPs or vaccines for both pre-clinical and

clinical trials, almost all drug development will face the same challenging obstacle of rapidly generating a high stable producer. Developing and identifying a stable cell line is a critical part of the development. However, to generate a stable cell line with high titer and good product quality is not so easily accomplished until now.

[0005] Chikungunya virus (CHIKV) has infected millions of people in Africa, Europe and Asia since this alphavirus reemerged from Kenya in 2004. The severity of the disease and the spread of this epidemic virus present a serious public health threat in the absence of vaccines or antiviral therapies. It is reported that a VLP vaccine for epidemic Chikungunya virus protects non-human primates against infection (Nat Med. 2010 March; 16(3): 334-338). US patent publication No. 2012/0003266 discloses a virus-like particle (VLP) comprising one or more Chikungunya viral structural proteins which is useful for formulating a vaccine or antigenic composition for Chikungunya that induces immunity to an infection or at least one symptom thereof. WO2012/106356 discloses modified alphavirus or flavivirus virus-like particles (VLPs) and methods for enhancing production of modified VLPs for use in the prevention or treatment of alphavirus and flavivirus-mediated diseases. (these cited references are herein incorporated by reference).

Citation List

Patent Literature

[0006] [PTL1]US2012/0003266

[PTL2]WO2012/106356

Non Patent Literature

[0007] [NPL1]Expert Rev. Vaccines 9(10), 1149-1176, 2010

[NPL2]Nat Med. 2010 March; 16(3): 334-338

Summary of Invention

[0008] According to the present application, followings are provided:

(1) A virus like particle comprising a viral structural protein which comprises modified envelope protein E3.

(2) The virus like particle according to (1), wherein the viral structural protein is derived from alphavirus or flavivirus.

(3) The virus like particle according to (2), wherein the viral structural protein is derived from Chikungunya virus or Venezuelan equine encephalitis virus.

(4) The virus like particle according to (3), wherein the virus like particle is derived from Chikungunya virus strain 37997 or strain OPY-1, or Venezuelan equine encephalitis virus strain TC-83.

(5) The virus like particle according to any one of (1)-(4), wherein one or more amino acid residues in the envelope protein E3 are replaced, added and/or deleted in

amino acid sequence of the viral structural protein.

(6) The virus like particle according to (5), wherein one or more amino acid residues are replaced, added and/or deleted in amino acid sequence at furin site in the envelope protein E3.

(7) The virus like particle according to any one of (1)-(6), wherein the viral structural protein comprises capsid, envelope protein E1, envelope protein E2 and envelope protein E3.

(8) The virus like particle according to any one of (1)-(7), wherein an at least one antigen is inserted into the envelope protein E3.

(9) The virus like particle according to (8), wherein the at least one antigen is further inserted into the envelope protein E2.

(10) The virus like particle according to (8) or (9), wherein the at least one antigen is inserted between residues corresponding to 321 and 326 of SEQ ID NO: 1, residues 321 and 326 of SEQ ID NO: 2 or residues 330 and 335 of SEQ ID NO: 3.

(11) The virus like particle according to any one of (8)-(10), wherein the at least one antigen is derived from Plasmodium falciparum circumsporozoite protein, PD-1, PD-L1, CTLA-4, DISC1, IL-2, HER2, BTLA or HVEM.

(12) The virus like particle according to (11), wherein a peptide selected from (NPNA)n (n=4-30), amino acid sequence represented by SEQ ID Nos.6-9 and 15-29 is inserted into the envelope E3 protein.

(13) The virus like particle according to any one of (8)-(12), wherein the virus like particle is Chikungunya virus like particle comprises the following amino acid sequences or an amino acid sequence having 90% or more identity to the following sequences:

a capsid which consists of an amino acid sequence represented by SEQ ID NO: 31 or SEQ ID NO: 75;

an E1 which consists of an amino acid sequence represented by SEQ ID NO: 32 or SEQ ID NO: 76; and

a complex of E2 and E3 which consists of an amino acid sequence represented by SEQ ID NO: 33 or SEQ ID NO: 77, wherein an amino acid sequence of the at least one antigen is inserted between residues corresponding to residues 321 and 326 of SEQ ID NO: 2.

(14) The virus like particle according to any one of (8)-(12), wherein the virus like particle is Venezuelan equine encephalitis virus like particle comprises the following amino acid sequences or an amino acid sequence having 90% or more identity to the following sequences:

a capsid which consists of an amino acid sequence represented by SEQ ID NO: 35;

an E1 which consists of an amino acid sequence represented by SEQ ID NO: 36; and

a complex of E2 and E3 which consists of an amino acid sequence represented by SEQ ID NO: 37, wherein an amino acid sequence of the at least one antigen is inserted between residues corresponding to the residues 330 and 335 of SEQ ID NO: 3.

(15) The virus like particle according to any one of (8)-(12), wherein the virus like particle is Chikungunya virus like particle and the structure of the virus like particle comprises any one of the following sequences (1)-(4) or an amino acid sequence having 90% or more identity to any one of the following sequences (1)-(4):

(1)

an E1 which consists of an amino acid sequence represented by SEQ ID NO: 32;

a complex of E2 and E3 into which a malaria antigen is inserted, which consists of an amino acid sequence represented by SEQ ID NO: 46; and

and a capsid which consists of an amino acid sequence represented by SEQ ID NO: 31;

(2)

an E1 which consists of an amino acid sequence represented by SEQ ID NO: 32;

a complex of E2 and E3 into which a PD-1 antigen is inserted, which consists of an amino acid sequence represented by SEQ ID NO: 47; and

a capsid which consists of an amino acid sequence represented by SEQ ID NO: 31;

(3)

an E1 which consists of an amino acid sequence represented by SEQ ID NO: 32;

a complex of E2 and E3 into which a PD-L1 ligand antigen is inserted, which consists of an amino acid sequence represented by SEQ ID NO: 48; and

a capsid which consists of an amino acid sequence represented by SEQ ID NO: 31; or

(4)

an E1 which consists of an amino acid sequence represented by SEQ ID NO: 32;

a complex of E2 and E3 into which a CTLA-4 antigen is inserted, which consists of an amino acid sequence represented by SEQ ID NO: 49; and

a capsid which consists of an amino acid sequence represented by SEQ ID NO: 31.

(16) The virus like particle according to any one of (8)-(12), wherein the virus like particle is Venezuelan equine encephalitis virus like particle and the structure of the virus like particle comprises any one of the following sequences (1)-(4) or an amino acid sequence having 90% or more identity to any one of the following sequences (1)-(4):

(1)

an E1 which consists of an amino acid sequence represented by SEQ ID NO: 36;

a complex of E2 and E3 into which a malaria antigen is inserted, which consists of an amino acid sequence represented by SEQ ID NO: 50; and

a capsid which consists of an amino acid sequence represented by SEQ ID NO: 35;

(2)

an E1 which consists of an amino acid sequence represented by SEQ ID NO: 36;
a complex of E2 and E3 into which a PD-1 antigen is inserted, which consists of an amino acid sequence represented by SEQ ID NO: 51; and
a capsid which consists of an amino acid sequence represented by SEQ ID NO: 35;
(3)
an E1 which consists of an amino acid sequence represented by SEQ ID NO: 36;
a complex of E2 and E3 into which a PD-L1 antigen is inserted, which consists of an amino acid sequence represented by SEQ ID NO: 52; and
a capsid which consists of an amino acid sequence represented by SEQ ID NO: 35; or
(4)
an E1 which consists of an amino acid sequence represented by SEQ ID NO: 36;
a complex of E2 and E3 into which a CTLA-4 antigen is inserted, which consists of an amino acid sequence represented by SEQ ID NO: 53; and
a capsid which consists of an amino acid sequence represented by SEQ ID NO: 35.

(17) The virus like particle according to any one of (1)-(5), wherein furin cleavage site located in envelope protein E3 is altered or mutated to prevent the furin site from cleaving.

(18). An isolated nucleic acid molecule comprising a nucleotide sequence encoding the virus like particle according to any one of (1-17).

(19) An isolated nucleic acid molecule consisting of a nucleotide sequence which has a sequence identity of 90% or more with a nucleotide sequence represented by any one of SEQ ID Nos.38-45.

(20) The nucleic acid molecule according to (19), wherein the nucleic acid molecule consists of a nucleotide sequence represented by any one of SEQ ID Nos.:38-45.

(21) A vector comprising the nucleic acid molecule according to any one of (18)-(20), wherein the vector optionally comprises an expression control sequence operably linked to the nucleic acid molecule.

(22) A vector comprising a nucleic acid molecule which comprises:
a nucleotide sequence represented by SEQ ID NO: 54 or SEQ ID NO: 55, wherein a nucleotide sequence encoding at least one antigen is inserted between residues 963 and 969 of SEQ ID NO: 54 or between residues 990 and 1006 of SEQ ID NO: 55; and
an expression control sequence operably linked to the nucleic acid molecule.

(23) A pharmaceutical composition comprising
(a) the virus like particle according to any one of (1)-(17), the nucleic acid molecule according to any one of (18)-(20) and/or the vector according to (21) or (22); and
(b) a pharmaceutically acceptable carrier.

(24) A vaccine composition comprising the virus like particle according to any one of (1)-(17) and a pharmaceutically acceptable carrier.

(25) A method of producing the virus like particle according to any one of (1)-(17), comprising the steps of:
culturing a cell which is transfected with the vector according to (21)-22) to express the virus like particle; and
purifying the generated particle.
(26) A method of enhancing the production of a virus like particle comprising a viral structural protein and at least one antigen, comprising
(1) inserting the at least one antigen into an envelope protein E3 of the viral structural protein, and
(2) isolating the virus like particle wherein at least one antigen is inserted into the envelope protein E3 of the viral structural protein.
(27) The method according to (26), wherein the step (1) is achieved by a method comprising preparing a nucleic acid molecule comprising a nucleotide sequence encoding the virus like particle wherein at least one antigen is inserted into the envelope protein E3 of the viral structural protein; and allow the nucleic acid molecule to be expressed using cells.
(28) The method according to (26) or (27), wherein the virus like particle is the virus like particle according to any one of (1)-(17).
(29) A method of treating or preventing cancer, neurological disease, infectious disease or malaria; producing an antibody against the at least one antigen in a mammal; modulating an immune response; immunostimulation; inhibiting function of the at least one antigen; or presenting an antigen on macrophage, comprising administering the virus like particle according to any one of (1)-(17), the nucleic acid molecule according to any one of (18)-(20), the vector according to (21) or (22) and/or the composition according to (23) or (24) to a subject in need thereof.
(30) Use of the virus like particle according to any one of (1)-(17), the nucleic acid molecule according to any one of (18)-(20) or the vector according to (21) or (22) for the manufacture of a pharmaceutical composition or a kit for treating or preventing cancer, infectious disease or malaria; producing an antibody against the at least one antigen in a mammal; modulating an immune response; immunostimulation; inhibiting function of the at least one antigen; or presenting an antigen on macrophage.
(31) A cell line expressing a virus like particle, wherein the virus like particle comprises a viral structural protein which comprises an alternation/mutation to the amino acid sequence at the furin site in the envelope protein E3.
(32) The cell line of (31), the alternation to the amino acid sequence at furin site is an alternation to Ile-Glu/Asp-Gly-Arg or Asp-Asp-Asp-Asp-Lys.
(33) The cell line of (31) or (32), wherein the cell line is a stable cell line.
(34) A method for producing a cell line expressing a virus like particle, which

comprising the step of:

transfecting a cell line with an expression vector comprising a nucleic acid molecule encoding a viral structural protein whose furin site in an envelope protein E3 is altered to a specific protease recognition site.

(35) The method of (34), wherein the furin site is altered to Factor Xa or Enterokinase recognition site.

(36) The method of (34) or (35), wherein the method provides a stable cell line.

(37) A method for producing a mature virus like particle, which comprises the steps of:

i) generating an immature virus like particle produced by the cell line according to any one of (31)-(33); and

ii) removing the E3 from the immature virus like particle.

(38) The method of (37), wherein the E3 is removed by a protease.

(39) The method of (38), wherein the protease is Factor Xa or Enterokinase.

(40) The cell line of (31), wherein the virus like particle is the virus like particle according to any one of (1)-(17).

[0009] In a first aspect, the present application provides a virus like particle comprising a modified envelope protein E3.

[0010] In a second aspect, the present application provides a nucleic acid molecule comprising or consisting of a nucleotide sequence encoding a virus like particle comprising a modified envelope protein E3.

[0011] In a third aspect, the present application provides a pharmaceutical composition and a kit comprising the pharmaceutical composition, wherein the pharmaceutical composition comprises (i) a virus like particle comprising a modified envelope protein E3 and/or (ii) a nucleic acid molecule comprising or consisting of a nucleotide sequence encoding a virus like particle comprising a modified envelope protein E3.

[0012] In a fourth aspect, the present application provides a method of producing a virus like particle comprising a modified envelope protein E3, comprising culturing a cell which is transfected with a vector to express the virus like particle; and purifying the particle generated by the cell.

[0013] In a fifth aspect, the present application provides a method of enhancing the production of a virus like particle comprising a viral structural protein with a modified envelop protein E3. In one embodiment, the virus like particle comprises a viral structural protein and at least one foreign antigen, comprising:

(1) inserting the at least one foreign antigen into an envelope protein E3 of the viral structural protein, and

(2) isolating the virus like particle wherein at least one antigen is inserted into the envelope protein E3 of the viral structural protein.

[0014] In a sixth aspect, the present application provides use of (i) a virus like particle

comprising a modified envelope protein E3 and/or (ii) a nucleic acid molecule consisting of a nucleotide sequence encoding a virus like particle comprising a modified envelope protein E3 for the manufacture of a pharmaceutical composition or a kit for treating or preventing cancer, neurological disease, infectious disease or malaria; producing an antibody against at least one antigen in a mammal; modulating an immune response; immunostimulation; inhibiting function of at least one antigen; or presenting an antigen on macrophage.

[0015] In all aspect, the envelope protein E3 may be modified to comprise at least one antigen or an alternation/mutation to the amino acid sequence at the furin site.

[0016] In a seventh aspect, the present application provides a cell line that expresses a viral structural protein and can generate virus like particle, wherein the viral structural protein comprises an alternation/mutation to the amino acid sequence at the furin site, and the method for producing thereof.

[0017] In an eighth aspect, the present application provides a method for producing a mature virus like particle, which comprises the steps of:

i) generating an immature virus like particle produced by the cell line described above;

ii) removing the E3 from the immature virus like particle.

Brief Description of Drawings

[0018] [fig.1]Figure 1 shows structure of CHIKV or VEEV viral structural protein and sequences of 6K.

[fig.2]Figure 2 shows representative structure of expression vector for a viral structural protein with modified E3 and E2 envelope proteins.

[fig.3]Figure 3 shows results of western blotting. A CSP repeat sequence of NPNA, which is an antigen derived from plasmodium falciparum circumsporozoite protein, was inserted into E2 or E3 of virus like particle of Venezuelan equine encephalitis virus (VEEV) (strain TC-83). The antigen-inserted VEEV virus like particle expressed in 293F cells was confirmed by western blotting using mouse anti-VEEV antibody (1:1000). 74: Malaria CSP repeat antigen (repeat x6)76: Malaria CSP repeat antigen (repeat X14)78: Malaria CSP repeat antigen (repeat X25)In the figure, “74” indicates that inserted antigen is 6XNPNA, “76” indicates that inserted antigen is 14XNPNA, and “78” indicates that inserted antigen is 25XNPNA; and “21” indicates that the antigen is inserted in E2 and “26” indicates that the antigen is inserted in E3.

[fig.4]Figure 4 shows results of western blotting. A mouse malaria CSP repeat antigen was inserted into E2 or E3 of virus like particle of Venezuelan equine encephalitis virus (VEEV) (strain TC-83). The antigen-inserted VEEV virus like particle expressed in 293F cells was confirmed by western blotting using mouse anti-VEEV antibody. In

the figure, "261.25" indicates VEEV VLP comprising E2 into which mouse malaria CSP repeat antigen was inserted, and "261.66" indicates VEEV VLP comprising E3 into which mouse malaria CSP repeat antigen was inserted.

[fig.5]Figure 5 shows results of western blotting. A CSP repeat sequence, malaria pfs25 domain 1 epitope or malaria pfs25 domain 2 epitope was inserted into E2 or E3 of virus like particle of Venezuelan equine encephalitis virus (VEEV) (strain TC-83). The antigen-inserted VEEV virus like particle expressed in 293F cells was confirmed by western blotting using mouse anti-VEEV antibody. In the figure, "74" indicates that inserted antigen is malaria CSP repeat epitope, "302R" indicates that inserted antigen is malaria pfs25 domain1 epitope, and "303R" indicates that inserted antigen is malaria pfs25 domain2 epitope; and "21" indicates that the antigen is inserted in E2 and "26" indicates that the antigen is inserted in E3.

[fig.6]Figure 6 shows results of western blotting. PD-1 epitope or PD-L1 epitope was inserted into E2 or E3 of virus like particle of Chikungunya virus (CHIKV) (strain 37997). The antigen-inserted CHIKV virus like particle expressed in 293F cells was confirmed by western blotting using mouse anti-CHIKV antibody. In the figure, "274.11" indicates CHIKV VLP comprising E2 into which PD-1 epitope was inserted, "274.56" indicates CHIKV VLP comprising E3 into which mouse PD-1 epitope was inserted, "299.15" indicates CHIKV VLP comprising E2 into which PD-L1 epitope was inserted, and "299.56" indicates CHIKV VLP comprising E3 into which mouse PD-L1 epitope was inserted.

[fig.7]Figure 7 shows results of western blotting. Malaria CSP repeat epitope was inserted into E2 or E3 of virus like particle of Chikungunya virus (CHIKV) (strain 37997). The antigen-inserted CHIKV virus like particle expressed in 293F cells was confirmed by western blotting using mouse anti-CHIKV antibody. In the figure, "74.11" indicates CHIKV VLP comprising E2 into which malaria CSP repeat epitope was inserted, "74.16" indicates CHIKV VLP comprising E3 into which mouse malaria CSP epitope was inserted.

[fig.8]Figure 8 shows results of western blotting regarding CHIKV immature construct from transient transfection.

[fig.9]Figure 9 shows results of western blotting regarding VEEV immature construct from transient transfection.

[fig.10]Figure 10 shows results of western blotting indicating that DISC1_451, _452 and _454-inserted VLPs were produced when those antigens were inserted into E3 and into both E2 and E3 (dual).

[0019] [fig.11]Figures 11 show results of western blotting indicating that human IL-2 wild type, human IL-2 mutant, mouse IL-2 wild type and mouse IL-2 mutant-inserted VLPs were produced when those antigens were inserted into E3.

[fig.12]Figure 12 shows results of western blotting indicating that human IL-2 wild type, human IL-2 mutant, mouse IL-2 wild type and mouse IL-2 mutant-inserted VLPs were produced when those antigens were inserted into E3.

[fig.13]Figure 13 shows results of the PCR, indicating that among 10 mice immunized with Control VLP, 9 mice were infected with malaria; among 10 mice immunized with Chikungunya VLP comprising Malaria CSP 4X repeat inserted into both envelop proteins E2 and E3 (261.261 VLP), 9 mice were not infected with malaria; and among 10 mice immunized with Chikungunya VLP comprising Malaria CSP 14X repeat inserted into both envelop proteins E2 and E3, 9 mice were not infected with malaria.

[fig.14]Figure 14 shows results of ELISA, indicating that E3-inserted as well as E2- and E3-inserted VLPs have higher titer than E2-inserted VLP.

[fig.15]Figures 15 shows western blotting indicating that hHER2-inserted VLPs were produced.

[fig.16]Figure 16 shows results of ELISA, indicating that E2- and E3-inserted VLP has higher titer than E2-inserted VLP.

[fig.17]Figure 17 shows that monoclonal antibody was obtained by using CHIKV-VLP comprising hBTLA antigen.

[fig.18]Figure 18 shows result of western blotting indicating that human hHVEM inserted VLPs were produced when those antigens were inserted into E3.

[fig.19]Figure 19 shows that a cell line that express immature Chikungunya VLP whose furin site in the E3 envelope protein is modified could generate the immature VLP even after 3 months culture.

[fig.20]Figure 20 shows that mouse PD-L1 inserted VLP was effectively protect mouse from tumor.

[fig.21]Figure 21 shows result Western Blot indicating that CHIKV-VLP CSP repeat antigen inserted VLPs were prepared.

[fig.22]Figure 22 shows malaria CSP repeat antigen 76 inserted VLP stimulated the production of anti CSP antigen antibodies in mice.

Description of Embodiments

[0020] (1) Virus like particle comprising a modified envelope protein E3

In a first aspect, the present application provides a virus like particle comprising a modified envelope protein E3.

[0021] In this aspect, the envelope protein E3 may be modified to comprise at least one antigen or an alternation/mutation to the amino acid sequence at the furin site (Arg-X-X-Arg).

[0022] The term “Arg-X-X-Arg” indicates the minimal cleavage site of furin and “X-X” includes any two amino acids.

[0023] A virus like particle is composed of one or more viral structural proteins that spontaneously assemble into a particulate structure.

[0024] A viral structural protein used for the present application may be any viral structural protein as long as it expresses a furin site.

[0025] In a seventh aspect, the present application provides a cell line, especially stable cell line expressing a virus like particle, wherein the virus like particle comprises an alternation/mutation to the amino acid sequence at the furin site (Arg-X-X-Arg), and the method for producing thereof. Example of the alternation to the amino acid sequence at furin site includes the alternation to Ile-Glu/Asp-Gly-Arg or Asp-Asp-Asp-Asp-Lys.

[0026] In one embodiment, the present application provides a method for producing a cell line expressing a virus like particle, wherein the furin site of the virus like particle is altered to a protease recognition site. In this embodiment, the cell line generated by this method could be a stable cell line. For example, the stable cell line obtained by this embodiment may maintain the ability to express and generate the VLP for relatively long time, such as more than three months.

[0027] In one embodiment, the present application provides an immature virus like particle produced by the cell line described above.

[0028] In an eighth aspect, the present application provides a method for producing a mature virus like particle, which comprises the steps of:

i) providing an immature virus like particle produced by the cell line described above;

ii) removing the E3 from the immature virus particle.

[0029] In one embodiment, the E3 in the immature virus like particle is removed by digestion of the protease recognition site. In one embodiment, the E3 in the immature virus like particle is removed by a protease. Examples of proteases include, but not limited to, Arg-C proteinase, Asp-N endopeptidase, Asp-N endopeptidase + N-terminal Glu, BNPS-Skatole , Caspase1 to Caspase10, Chymotrypsin, Clostripain (Clostridiopeptidase B), CNBr, Enterokinase, Factor Xa, Formic acid, Glutamyl endopeptidase, GranzymeB, Hydroxylamine, Iodosobenzoic acid, LysC, LysN, NTCB (2-nitro-5-thiocyanobenzoic acid), Neutrophil elastase, Pepsin, Proline-endopeptidase , PreScission Protease (PSP), Proteinase K, Staphylococcal peptidase I, Tobacco etch virus protease , Thermolysin, Thrombin and Trypsin. Preferred examples of the protease include Factor Xa, Enterokinase and PreScission Protease (PSP), expecially, Factor Xa and Enterokinase (e.g. Enterokinase (enteropeptidase), light chain).

[0030] In another aspect, the present application provides a virus like particle comprising an envelope protein E3, wherein the envelope protein E3 is modified to comprise at least one antigen. The at least one antigen may be a peptide that is not derived from the virus from which the viral structural protein is derived or a peptide that is derived from

the same virus that provides the viral structural protein.

[0031] A derivative of the above-described virus like particle which can be prepared by modifying the above-described particle is also provided by the present application. Examples of the modification include, but are not limited to, addition, deletion or replacement of one or more amino acid residues.

[0032] The particle provided by the present application may be a particle which consists of or comprises i) at least one viral structural protein and ii) at least one antigen, wherein the at least one antigen is inserted into the envelope protein E3 of the viral structural protein. The at least one viral structural protein may consist of one or more kinds of protein or peptide and spontaneously assembles to form a particle. In one embodiment, the particle provided by the present application has a diameter of at least 10nm, for example, at least 20nm, preferably at least 50nm. In one embodiment, molecular weight of the particle is from 100 kDa to 100,000 kDa, preferably from 400kDa to 30,000kDa.

[0033] One or more amino acid residues can be replaced, added and/or deleted in amino acid sequence of the viral structural protein to allow expression of a virus like particle comprising an envelope protein E3 where at least one antigen is inserted.

[0034] In one preferred embodiment, the virus like particle provided by the present application comprising at least one antigen in an envelope protein E3 can be expressed more efficiently in a eukaryotic cell (e.g. 293F cells) than a virus like particle comprising said at least one antigen in an envelope protein E2.

[0035] A viral structural protein used for the present application may be a viral structural protein derived from Alphavirus or Flavivirus. Thus, the particle provided by the present application may be a virus like particle including a virus like particle derived from Alphavirus or Flavivirus.

[0036] Examples of Alphavirus and Flavivirus include, but not limited to, Aura virus, Babanki virus, Barmah Forest virus (BFV), Bebaru virus, Cabassou virus, Chikungunya virus (CHIKV), Eastern equine encephalitis virus (EEEV), Eilat virus, Everglades virus, Fort Morgan virus, Getah virus, Highlands J virus, Kyzylagach virus, Mayaro virus, Me Tri virus, Middelburg virus, Mosso das Pedras virus, Mucambo virus, Ndumu virus, O'nyong-nyong virus, Pixuna virus, Rio Negro virus, Ross River virus (RRV), Salmon pancreas disease virus, Semliki Forest virus, Sindbis virus, Southern elephant seal virus, Tonate virus, Trocara virus, Una virus, Venezuelan equine encephalitis virus (VEEV), Western equine encephalitis virus (WEEV),Whataroa virus, West Nile virus, dengue virus, tick-borne encephalitis virus and yellow fever virus.

[0037] The particle provided by the present application may be a virus like particle derived from Chikungunya virus or Venezuelan equine encephalitis virus. Chikungunya virus

may be Chikungunya virus 37997 strain or OPY-1 strain. Venezuelan equine encephalitis virus may be Venezuelan equine encephalitis virus TC-83 strain.

[0038] Viral structural protein may be a capsid protein, an envelope protein, a fragment thereof or a complex thereof. Thus, viral structural protein used for the present application may consist of or comprise a capsid protein and/or an envelope protein and/or a fragment or derivative thereof. In one embodiment, the virus like particle provided by the present application consists of or comprises capsid, E3, E2 and E1 proteins, and an antigen is inserted into E3. For example, the virus like particle provided by the present application may be formed by assembling 240 capsids, 240 E1 proteins, 240 E2 proteins and 240 E3 proteins where an antigen is inserted into each of E3 proteins.

[0039] Under physiological conditions, E3 can be dissociated from E2 after furin cleavage. In one embodiment, the furin cleavage site located in E3 may be mutated to prevent furin site from cleaving. For example, an antigen can be inserted into the furin cleavage site to introduce a mutation in the furin cleavage site. In this embodiment, the virus like particle provided may consist of or comprises capsid, E3, E2 and E1 proteins, where E3 is bound to E2 to form a single protein and an antigen is inserted into E3 region. For example, the virus like particle provided by the present application may be formed by assembling 240 capsids, 240 E1 proteins, 240 proteins in each of which E2 is bound to E3 and an antigen is inserted into each of E3 regions.

[0040] Antigen may be a molecule capable of being bound by an antibody or a T cell receptor (TCR) if it is presented with MHC molecules. Antigen can encompass B-cell epitopes and T-cell epitopes. Antigen disclosed in U.S. patent publication No.: US 2013/0251744 filed February 15, 2013, the entire contents of which are incorporated herein by reference, may be used for the present application. Examples of antigen used for the present application include, but are not limited to, allergens, self-antigens, haptens, cancer antigens, infectious disease antigens and small organic molecules, and fragments and derivatives thereof.

[0041] Antigen may be a naturally occurring and/or modified protein, a fragment thereof or derivative of the naturally occurring protein or its fragment. A fragment of a naturally occurring and/or modified protein for use as an antigen contained in the particle provided by the present application may be selected based on the amino acid sequence of the naturally occurring and/or modified protein and/or tertiary structure thereof. For example, a fragment for use as an antigen may consist of or comprise a fragment located in the surface of a naturally occurring protein. Preferably, an antibody against an antigen contained in the particle provided by the present application may inhibit function of the antigen. An antigen (e.g. a fragment of a naturally occurring protein) may be 10-300 amino acid residues (e.g. 10-120, 10-30 or 15-30 amino acid residues) in length. A derivative of a naturally occurring protein or its fragment may be prepared

by addition, deletion or replacement of one or several amino acid residues in the naturally occurring protein or its fragment. In one embodiment, a derivative of a naturally occurring protein or its fragment has at least 70%, 75%, 80%, 85%, 90%, 95% or 98% amino acid sequence identity to the corresponding naturally occurring protein or its fragment. In one embodiment, a derivative of a naturally occurring protein or its fragment is a mutant where at most 10% of the amino acids are deleted, substituted, and/or added based on the corresponding naturally occurring protein or its fragment.

[0042] In one embodiment, an antigen or epitope (e.g. a fragment of a naturally occurring protein) may be selected so that spatial distance between the N-terminal residue and C-terminal residue of the antigen is 30 angstroms or less when the distance is determined in a crystal of the antigen or a naturally occurring protein containing the antigen or modified peptide therefrom. For example, an antigen used for the particle provided by the present application can be designed using a free software including PyMOL (e.g. PyMOL v0.99: http:/www.pymol.org). In one embodiment, a spatial distance between N-terminal residue and C-terminal residue of the antigen is 30 angstroms or less, 20 angstroms or less, or 10 angstroms or less (e.g. from 5 angstroms to 15 angstroms, from 5 angstroms to 12 angstroms, from 5 angstroms to 11 angstroms, from 5 angstroms to 10 angstroms, from 5 angstroms to 8 angstroms, from 8 angstroms to 15 angstroms, from 8 angstroms to 13 angstroms, from 8 angstroms to 12 angstroms, from 8 angstroms to 11 angstroms, from 9 angstroms to 12 angstroms, from 9 angstroms to 11 angstroms, from 9 angstroms to 10 angstroms or from 10 angstroms to 11 angstroms).

[0043] In one embodiment, an antigen or epitope which may be used for the present application may be malaria antigen, PD-1 antigen, PD-1 ligand antigen, CTLA-4 antigen, IL-2 antigen DISC1 antigen, HER2 antigen, BTLA antigen, HVEM antigen, PCSK9 antigen or DPP-4 antigen.

[0044] As used herein, "malaria antigen" refers to an antigen or epitope derived from Plasmodium parasite. The plasmodium parasite may be selected from any of the known Plasmodium (P.) species, for example, P. falciparum, P. malariae, P. ovale, P. vivax, P. knowlesi, P. berghei, P. chabaudi and P. yoelii.

[0045] In one embodiment, malaria antigen is a Plasmodium falciparum circumsporozoite protein B cell epitope (hereinafter, referred as CSP protein, Malaria CSP protein, or CSP). Example of Plasmodium falciparum circumsporozoite protein B cell epitope may be a repeat sequence of NPNA, including (NPNA)4-30 (i.e. 4xNPNA, 5xNPNA, 6xNPNA, 7xNPNA, 8xNPNA, 9xNPNA, 10xNPNA, 11xNPNA, 12xNPNA, 13xNPNA 14xNPNA, 15xNPNA, 16xNPNA, 17xNPNA, 18xNPNA, 19xNPNA, 20xNPNA, 21xNPNA, 22xNPNA, 23xNPNA, 24xNPNA, 25xNPNA, 26xNPNA,

27xNPNA, 28xNPNA, 29xNPNA or 30xNPNA).

[0046] In one embodiment, malaria antigen is a Plasmodium yoelii circumsporozoite protein B cell epitope including (QGPGAP)3-12.

[0047] In one embodiment, malaria antigen is a Plasmodium vivax circumsporozoite protein B cell epitope including (ANGAGNQPG)1-12.

[0048] In one embodiment, malaria antigen is a Plasmodium malariae circumsporozoite protein B cell epitope including (NAAG)4-30.

[0049] In one embodiment, malaria antigen is a Plasmodium falciparum circumsporozoite protein T cell epitope. Example of Plasmodium falciparum circumsporozoite protein T cell epitope may be EYLNKIQNSLSTEWSPCSVT (SEQ ID NO: 4). (EYLNKIQNSLSTEWSPCSVT)1-6 may be also used as a malaria antigen.

[0050] In one embodiment, malaria antigen is a Plasmodium yoelii circumsporozoite protein T cell epitope which is YNRNIVNRLLGDALNGPEEK (SEQ ID NO: 5). (YNRNIVNRLLGDALNGPEEK)1-6 may be also used as a malaria antigen.

[0051] As used herein, the term "PD-1 antigen" refers to an antigen or epitope derived from PD-1. Preferably, PD-1 is a human PD-1. An antigen derived from PD-1 may be a fragment of PD-1 or a derivative of a fragment of PD-1.

[0052] As used herein, the term "PD-1 ligand antigen" refers to an antigen or epitope derived from a ligand of PD-1. Examples of a ligand of PD-1 include, but are not limited to, PD-L1 and PD-L2. Preferably, a ligand of PD-1 is human PD-L1 or human PD-L2. An antigen derived from PD-L1 may be a fragment of PD-L1 or PD-L2; or a derivative of a fragment of PD-L1 or PD-L2.

[0053] Examples of PD-1 antigen for use as an antigen include, but are not limited to, lnwyrmspsnqtdklaaf (SEQ ID NO: 6), mlnwyrmspsnqtdklaafs (SEQ ID NO: 7), vl-nwyrmspsnqtdklaafp (SEQ ID NO: 8), gaislhpkakiees (SEQ ID NO: 9), cgaislh-pkakieec (SEQ ID NO: 10), VLNWYRMSPSNQTDKLAAF (SEQ ID NO: 11), GAIS-LAPKAQIKES (SEQ ID NO: 12), RNDSGTYLCGAISLAPKAQIKESLRAELRVT (SEQ ID NO: 13) and RNDSGIYLCGAISLHPKAKIEESPGAELVVT (SEQ ID NO: 14). Examples of PD-1 ligand antigen for use as an antigen include, but are not limited to, ciisyggadyc (SEQ ID NO: 15), CMISYGGADYC (SEQ ID NO: 16), LQDAGVYRCMISYGGADYKRITVKVN (SEQ ID NO: 17), LQDAGVYRA-MISYGGADYKRITVKVN (SEQ ID NO: 18), DLAALIVYWEMEDKNIIQFVH (SEQ ID NO: 19), DLAALIVYWEMEDKNIIQFVHGG (SEQ ID NO: 20), FTVTVP-KDLYVVEYGSNMTIECKFPVE (SEQ ID NO: 21), Lqdagvycciisyggadykritlkvn (SEQ ID NO: 22), lqdagvyaaiisyggadykritlkvn (SEQ ID NO: 23), dllalvvyweked-eqviqfva (SEQ ID NO: 24), dllalvvywekedeqviqfvagg (SEQ ID NO: 25) and ftitapkd-lyvveygsnvtmecrfpve (SEQ ID NO: 26).

[0054] As used herein, the term "CTLA-4 antigen" refers to an antigen or epitope derived

from CTLA-4 (cytotoxic T-lymphocyte-associated antigen 4). Preferably, CTLA-4 is a human CTLA-4. An antigen derived from CTLA-4 may be a fragment of CTLA-4 or a derivative of a fragment of CTLA-4.

Examples of CTLA-4 antigen for use as an antigen include, but are not limited to, ggkvelmypppyfvgmgg (SEQ ID NO: 27), cattftekntvgfldypfc (SEQ ID NO: 28) and at-tftekntvgfldypf (SEQ ID NO: 29).

[0055] As used herein, the term “DISC1 antigen" refers to an antigen or epitope derived from DISC1. Examples of DISC1 antigen for use as an antigen include, but are not limited to, SGGLLIQSLQLQEARGELSVEDERQMDDLEGGS (DISC1_451)(SEQ ID NO: 105),

SGGEARGELSVEDERQMDDLEGGS (DISC1_452)(SEQ ID NO: 106)

and

SGGEARGELSVEGGS (DISC1_454)(SEQ ID NO: 107).

[0056] As used herein, the term “HER2 antigen" refers to an antigen or epitope derived from HER2. Examples of HER2 antigen for use as an antigen include, but are not limited to, SGGVTYNTDTFESMPGGS (SEQ ID NO: 108), SGGEYVNARHCLPGGS(SEQ ID NO: 109), SGGYVNARHCLGGS(SEQ ID NO: 110), SGGYVNARHGLGGS(SEQ ID NO: 111), SGGKFPDEEGACQPCPIGGS(SEQ ID NO: 112), SGGKFPDEE-GACQPGGS(SEQ ID NO: 113), SGGKDPPFCVGGS(SEQ ID NO:114), SG-GYKDPPFCVAGGS(SEQ ID NO: 115), and SGGYKDPPFCVGGS(SEQ ID NO: 116).

[0057] As used herein, the term "BTLA antigen" refers to an antigen or epitope derived from BTLA. Examples of BTLA antigen for use as an antigen include, are not limited to, SGGCKLNGTTCGGS (SEQ ID NO: 132).

[0058] As used herein, the term "HVEM antigen" refers to an antigen or epitope derived from HVEM. Examples of HVEM antigen for use as an antigen include, are not limited to, SGGCVKEASGELTGTVCGGS (SEQ ID NO: 133), SGGCYRVKEAS-GELTGTVSEPCGGS (SEQ ID NO: 134), SGGCSRNSSRTENAVCGGS (SEQ ID NO: 135), and SGGCQMSDPAMGLRSRNCGGS (SEQ ID NO: 136).

[0059] In the particle as provided by the present application, a viral structural protein and an antigen may be linked through at least one first attachment site which is present in the viral structural protein and at least one second attachment site which is present in the antigen.

[0060] As used herein, each of "a first attachment site" and "a second attachment site" refers to a site where more than one substance is linked each other.

[0061] A viral structural protein and an antigen may be directly or indirectly fused. In one embodiment, one or two linkers may intervene between N-terminal residue of an antigen and a viral structural protein and/or between C-terminal residue of an antigen

and a viral structural protein.

[0062] An antigen or a viral structural protein can be truncated and replaced by short linkers. In some embodiments, an antigen or a viral structural protein include one or more peptide linkers. Typically, a linker consists of from 2 to 25 amino acids (e.g. 2, 3, 4, 5 or 6 amino acids). Usually, it is from 2 to 15 amino acids in length, although in certain circumstances, it can be only one, such as a single glycine residue.

[0063] In one embodiment, a nucleic acid molecule, in which polynucleotide encoding the viral structural protein is genetically fused with polynucleotide encoding the antigen, is expressed in a host cell (e.g. mammalian cells (e.g. 293F cells)) so that the first attachment site and the second attachment site are linked through a peptide bond. In this case, the viral structural protein and the antigen are linked through a peptide bond. Relating to this embodiment, the first attachment site and/or the second attachment site may be genetically modified from the original protein or antigen. For example, the first attachment site is modified from the viral structural protein so that through a linker peptide including SG, GS, SGG, GGS and SGSG, the protein is conjugated with the antigen. When the viral structural protein are chemically conjugated with the antigen, the first attachment site and the second attachment site may be linked through a chemical cross-linker which is a chemical compound. Examples of the cross-linker include, but are not limited to, SMPH, Sulfo-MBS, Sulfo-EMCS, Sulfo-GMBS, Sulfo-SIAB, Sulfo-SMPB, Sulfo-SMCC, SVSB, SIA and other cross-linkers available from the Pierce Chemical Company.

[0064] Preferably, an antigen may be linked to the Chikungunya viral structural protein or Venezuelan equine encephalitis viral structural protein as a fusion protein produced by way of genetic engineering.

[0065] A Chikungunya viral structural protein or Venezuelan equine encephalitis viral structural protein used in the present application may be a Chikungunya or Venezuelan equine encephalitis virus envelope protein or a capsid or a complex of one or more envelope proteins and/or a capsid protein.

[0066] Examples of Chikungunya virus include, but are not limited to, strains 37997 and strain LR2006 OPY-1.

Examples of Venezuelan equine encephalitis virus include, but are not limited to, strain TC-83.

[0067] Chikungunya viral structural protein or Venezuelan equine encephalitis viral structural protein used in the present application may be a naturally occurring viral structural protein or modified protein thereof. The modified protein may be a fragment of the naturally occurring viral structural protein. In one embodiment, the modified protein has at least 70%, 75%, 80%, 85%, 90%, 95% or 98% amino acid sequence identity to a naturally occurring viral capsid and/or envelope protein. In one em-

bodiment, the modified protein is a mutant where at most 10% of the amino acids are deleted, substituted, and/or added based on a naturally occurring viral capsid and/or envelope protein. For example, K64A or K64N mutation may be introduced into a capsid of Venezuelan equine encephalitis viral structural protein used in the present application.

[0068] Chikungunya or Venezuelan equine encephalitis viral structural protein may consist of or comprise a capsid, E3, E2 and E1 proteins. E3 and E2 proteins may be expressed together so that E2 and E3 can form one protein.

[0069] Examples of Chikungunya viral structural protein include, but are not limited to, Capsid-E3-E2-E1 of Chikungunya virus Strain 37997, and Capsid-E3-E2-E1 of Chikungunya virus LR2006 OPY-1.

[0070] Examples of Venezuelan equine encephalitis viral structural protein include, but are not limited to, Capsid-E3-E2-E1 of Venezuelan equine encephalitis virus Strain TC-83.

[0071] An exemplary Chikungunya viral structural protein sequence is provided at Genbank Accession No. ABX40006.1, which is described below (SEQ ID NO: 1):

[Chem. 1]

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mefiptqtfynrryqprpwtprptiqvirprprpqrqagqlaqlisavnkltmravpqqkpr
rnrknkkqkqkqqapqnntnqkkqppkkkpaqkkkkpgrrermcmkiendcifevkhegkvt
gyaclvgdkvmkpahvkgtidnadlaklafkrsskydlecaqipvhmksdaskfthekpegy
ynwhhgavqysggrftiptgagkpgdsgrpifdnkgrvvaivlgganegartalsvvtwnkd
ivtkitpegaeewslaipvmcllanttfpcsqppctpccyekepeetlrmlednvmrpgyyq
llqasltcsphrqrrstkdnfnvykatrpylahcpdcgeghschspvalerirneatdgtlk
iqvslqigiktddshdwtklrymdnhmpadaeraglfvrtsapctitgtmghfilarcpkge
tltvgftdsrkishscthpfhhdppvigrekfhsrpqhgkelpcstyvqstaatteeievhm
ppdtpdrtlmsqqsgnvkitvngqtvrykcncggsneglttdkvinnckvdqchaavtnhk
kwqynsplvprnaelgdrkgkihipfplanvtcrvpkarnptvtygknqvimllypdhptll
syrnmgeepnyqeewvmhkkevvltvpteglevtwgnnepykywpqlstngtahghpheiil
yyyelyptmtvvvvsvatfillsmvgmaagmcmcarrrcitpyeltpgatvpfllsliccir
takaatyqeaaiylwneqqplfwlqaliplaalivlcnclrllpcccktlaflavmsvgaht
vsayehvtvipntvgvpyktlvnrpgyspmvlemellsvtleptlsldyitceyktvipspy
vkccgtaeckdknlpdysckvftgvypfmwggaycfcdaentqlseahveksescktefasa
yrahtasasaklrvlyqgnnitvtayangdhavtvkdakfivgpmssawtpfdnkivvykgd
vynmdyppfgagrpgqfgdiqsrtpeskdvyantqlvlqrpavgtvhvpysqapsgfkywlk
ergaslqhtapfgcqiatnpvravncavgnmpisidipeaaftrvvdapsltdmscevpact
hssdfggvaiikyaaskkgkcavhsmtnavtireaeievegnsqlqisfstalasaefrvqv
cstqvhcaaechppkdhivnypashttlgvqdisatamswvqkitggvglvvavaalilivv
lcvsfsrh
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[0072] Another exemplary Chikungunya viral structural protein sequence is provided at Genbank Accession No. ABX40011.1, which is described below (SEQ ID NO: 2):

[Chem. 2]

mefiptqtfynrryqprpwaprptiqvirprprpqrqagqlaqlisavnkltmravpqqkpr
rnrknkkqrqkkqapqndpkqkkqppqkkpaqkkkkpgrrermcmkiendcifevkhegkvm
gyaclvgdkvmkpahvkgtidnadlaklafkrsskydlecaqipvhmksdaskfthekpegy
ynwhhgavqysggrftiptgagkpgdsgrpifdnkgrvvaivlgganegartalsvvtwnkd
ivtkitpegaeewslalpvlcllanttfpcsqppctpccyekepestlrmlednvmrpgyyq
llkasltcsphrqrrstkdnfnvykatrpylahcpdcgeghschspialerirneatdgtlk
iqvslqigiktddshdwtklrymdshtpadaeragllvrtsapctitgtmghfilarcpkge
tltvgftdsrkishtcthpfhheppvigrerfhsrpqhgkelpcstyvqstaataeeievhm
ppdtpdrtlmtqqsgnvkitvngqtvrykcncggsneglttfdkvinnckidqchaavtnhk
nwqynsplvprnaelgdrkgkihipfplanvtcrvpkarnptvtygknqvtmllypdhptll
syrnmgqepnyheewvthkkevtltvpteglevtwgnnepykywpqmstngtahghpheiil
yyyelyptmtvvivsvasfvllsmvgtavgmcvcarrrcitpyeltpgatvpfllsllccvr
ttkaatyyeaaaylwneqqplfwlqaliplaalivlcnclkllpcccktlaflavmsigaht
vsayehvtvipntvgvpyktlvnrpgyspmvlemelqsvtleptlsldyitceyktvipspy
vkccgtaeckdkslpdysckvftgvypfmwggaycfcdaentqlseahveksecktefasa
yrahtasasaklrvlyqgnnitvaayangdhavtvkdakfvvgpmssawtpfdnkivvykgd
vynmdyppfgagrpgqfgdiqsrtpeskdvyantqlvlqrpaagtvhvpysqapsgfkywlk
ergaslqhtapfgcqiatnpvravncavgnipisidipdaaftrvvdapsvtdmscevpact
hssdfggvaiikytaskkgkcavhsmtnavtireadvevegnsqlqisfstalasaefrvqv
cstqvhcaaachppkdhivnypashttlgvqdisttamswvqkitggvglivavaalilivv
lcvsfsrh

[0073] An exemplary Venezuelan equine encephalitis viral structural protein is provided at Genbank Accession No. L01443.1 (http://www.ncbi.nlm.nih.gov/nuccore/L01443.1), which is described below (SEQ ID NO: 3):

[Chem. 3]

mfpfqpmypmqpmpyrnpfaaprrpwfprtdpflamqvqeltrsmanltfkqrrdappegps
aakpkkeasqkqkgggqgkkknqgkkkaktgppnpkaqngnkkktnkkpgkrqrmvmkles
dktfpimlegkingyacvvggklfrpmhvegkidndvlaalktkkaskydleyadvpqnmra
dtfkythekpqgyyswhhgavqyengrftvpkgvgakgdsgrpildnqgrvvaivlggvneg
srtalsvvmwnekgvtvkytpenceqwslvttmcllanvtfpcaqppicydrkpaetlamls
vnvdnpgydelleaavkcpgrkrrsteelfneykltrpymarcircavgschspiaieavks
dghdgyvrlqtssqygldssgnlkgrtmrydmhgtikeiplhqvslytsrpchivdghgyfl
larcpagdsitmefkkdsvrhscsvpyevkfnpvgrelythppehgveqacqvyahdaqnrg
ayvemhlpgsevdsslvslsgssvtvtppdgtsalvececggtkisetinktkqfsqctkke
qcrayrlqndkwvynsdklpkaagatlkgklhvpflladgkctvplapepmitfgfrsvslk
lhpknptylitrqladephythelisepavrnftvtekgwefvwgnhppkrfwaqetapgnp
hglphevithyyhrypmstilglsicaaiatvsvaastwlfcrsrvacltpyrltpnaripf
clavlccartaraettwesldhlwnnnqqmfwiqlliplaalivvtrllrcvccvvpflvma
gaagagayehattmpsqagisyntivnragyaplpisitptkikliptvnleyvtchyktgm
dspaikccgsqectptyrpdeqckvftgvypfmwggaycfcdtentqvskayvmksddclad
haeaykahtasvqaflnitvgehsivttvyvngetpvnfngvkitagplstawtpfdrkivq
yageiynydfpeygagqpgafgdiqsrtvsssdlyantnlvlqrpkagaihvpytqapsgfe
qwkkdkapslkftapfgceiytnpiraencavgsiplafdipdalftrvsetptlsaaectl
necvyssdfggiatvkysasksgkcavhvpsgtatlkeaavelteqgsatihfstanihpef
rlqictsyvtckgdchppkdhivthpqyhaqtftaavsktawtwltsllggsaviiiiglvl
ativamyvltnqkhn

[0074] In one embodiment, a first attachment site comprises an amino group, preferably an amino group of a lysine residue. In one embodiment, the second attachment site comprises sulfhydryl group, preferably, a sulfhydryl group of a cysteine.

[0075] According to the present application, a Chikungunya virus like particle or Venezuelan equine encephalitis virus like particle comprising a Chikungunya or Venezuelan equine encephalitis viral structural protein and at least one antigen, wherein the at least one antigen is inserted in E3 of the viral structural protein, and the Chikungunya viral structural protein or Venezuelan equine encephalitis viral structural protein and the antigen are expressed as a fusion protein can be provided. The antigen may be inserted directly or indirectly in E3 of the viral structural protein.

[0076] The viral structural protein of Chikungunya virus as well as Venezuelan equine encephalitis consist of E1, E2, 6K and E3. 6K is naturally cleaved during the process of assemble and removed from the VLPs. The mature VLPs consists of capsid, E1 and E2 (See Figure 1). In the present specification and claims, "viral structural protein" refers not only those having 6K but also after 6K is removed.

[0077] 6K sequences of the CHIKV and VEEV used in the working examples are as follows:

CHIKV OPY-1 Strain, 6K: 749-809aa of SEQ ID NO: 1
atyqeaaiylwneqqplfwlqaliplaalivlcnclrllpcccktlaflavmsvgahtvsa (SEQ ID NO: 137)
CHIKV 37997 strain, 6K: 749-809aa of SEQ ID NO: 2
atyyeaaaylwneqqplfwlqaliplaalivlcnclkllpcccktlaflavmsigahtvsa (SEQ ID NO: 138)
VEEV TC-83strain, 6K: 758-813aa of SEQ ID NO: 3
ettwesldhlwnnnqqmfwiqlliplaalivvtrllrcvccvvpflvmagaagaga
(SEQ ID NO: 139)

[0078] Regarding Chikungunya viral structural protein, at least one antigen may be inserted instead of furin site (RKRR) from 322R to 325R of SEQ ID NO: 1 or 2. For example, regarding Chikungunya viral structural protein, at least one antigen is inserted between residues H at 321-position and S at 326-position of SEQ ID NO: 1 or 2; between P at 320-position and S at 326-position of SEQ ID NO: 1 or 2; or between S at 319-position and S at 326-position of SEQ ID NO: 1 or 2. VLP_CHI 0.56 vector (SEQ ID NO: 30) may be used for preparing Chikungunya virus like particle where the antigen is inserted between residues 321 and 326 of SEQ ID Nos.1 or 2. When an antigen is inserted between residues 321 and 326 of SEQ ID Nos.1 or 2, the virus like particle provided by the present application may be Chikungunya virus like particle consisting of a complex of E2 and E3, capsid and E1, and wherein the at least one antigen is inserted into E3 region, and wherein the capsid consists of an amino acid sequence represented by SEQ ID NO: 31 (or SEQ ID NO: 75); the E1 consists of an amino acid sequence represented by SEQ ID NO: 32 or SEQ ID NO: 76; and the complex of E2 and E3 consists of an amino acid sequence represented by SEQ ID NO: 33 or SEQ ID NO: 77 provided that an amino acid sequence of the at least one antigen is inserted between residues corresponding to 321 and 326 of SEQ ID NO: 1 or 2.

[0079] Venezuelan equine encephalitis viral structural protein, at least one antigen may be inserted instead of furin site (RKRR) from 331R to 334R of SEQ ID NO: 3. For example, regarding Venezuelan equine encephalitis viral structural protein, at least one antigen is inserted between G at 330-position and S at 335-position of SEQ ID NO: 3; between P at 329-position and S at 335-position of SEQ ID NO: 3; or between C at 328-position and S at 335-position of SEQ ID NO: 3. VLP_VEEV 0.66 vector (SEQ ID No: 34) may be used for preparing Venezuelan equine encephalitis virus like particle where the antigen is inserted between residues 330 and 335 of SEQ ID NO: 3. When an antigen is inserted between residues 330 and 335 of SEQ ID NO: 3, the virus like particle provided by the present application may be Venezuelan equine encephalitis virus like particle consisting of a complex of E2 and E3, capsid and E1, and wherein the at least one antigen is inserted into E3 region, and wherein the capsid consists of an amino acid sequence represented by SEQ ID NO: 35; the E1 consists of an amino acid sequence represented by SEQ ID NO: 36; and the complex of E2 and E3 consists of an amino acid sequence represented by SEQ ID NO: 37 provided that an amino acid sequence of the at least one antigen is inserted between residues 330 and 335 of SEQ ID NO: 3.

[0080] In one embodiment, at least one antigen selected from an antigen derived from Plasmodium falciparum circumsporozoite protein, an antigen derived from PD-1, an antigen derived from PD-L1, an antigen derived from CTLA-4, an antigen derived from IL-2, an antigen derived from DISC1 or an antigen derived from HER2 is inserted into E3, an antigen derived from BTLA, an antigen derived from HVEM, an antigen derived from PCSK9 or an antigen derived from DPP-4 of Chikungunya viral structural protein or Venezuelan equine encephalitis viral structural protein.

[0081] The fusion protein may be expressed using a conventional technique in the art. A variety of expression systems can be used for the expression of the fusion protein. For example, the fusion protein can be expressed in 293F cells, Sf9 cells, E.coli, insect cell or Baculovirus.

[0082] A protein derived from Chikungunya virus (CHIKV) or Venezuelan equine encephalitis virus (VEEV) may be a naturally occurring viral protein or modified protein thereof.

[0083] When a protein derived from a virus is conjugated with a protein derived from an antigen, a linker peptide including SG, GS, SGG, GGS SGSG and TRGGS may be used. Examples of conjugation of the protein derived from a virus (referred to as "PFV" below) with the protein derived from the antigen (referred to as "PFA" below) include, but not limited to: PFV-SG-PFA-GS-PFV; PFV-SG-PFA-GGS-PFV; PFV-SSG-PFA-GS-PFV; PFV-SGG-PFA-GGS-PFV; PFV-SGSG-PFA-GS-PFV; and PFA-SGG-PFA-TRGGS-PFV.

[0084] In one embodiment, the present application provides a virus like particle comprising a fusion protein of a protein derived from Chikungunya virus (CHIKV) or Venezuelan equine encephalitis virus (VEEV) and a protein derived from Plasmodium falciparum circumsporozoite protein, PD-1, PD-L1, CTLA-4, an antigen derived from IL-2, an antigen derived from DISC1, an antigen derived from HER2, an antigen derived from BTLA, an antigen derived from HVEM, an antigen derived from PCSK9 and an antigen derived from DPP-4, wherein the virus like particle is prepared by transfecting an expression vector comprising a nucleic acid molecule comprising a nucleotide sequence represented by SEQ ID Nos.:38-45 into a mammalian cell (e.g. 293F cell). Regarding this embodiment, modified fusion protein can be prepared by transfecting an expression vector comprising a nucleic acid molecule comprising a nucleotide sequence having at least 70%, 75%, 80%, 85%, 90%, 95% or 98% amino acid sequence identity to SEQ ID Nos.: 38-45 into a mammalian cell (e.g. 293F cell).

[0085] In one embodiment, the present application provides a virus like particle comprising or consisting of:

one or more capsid of Chikungunya virus (CHIKV) or Venezuelan equine encephalitis virus (VEEV);

one or more E1 of Chikungunya virus (CHIKV) or Venezuelan equine encephalitis virus (VEEV);

one or more E2 of Chikungunya virus (CHIKV) or Venezuelan equine encephalitis virus (VEEV); and

one or more E3 of Chikungunya virus (CHIKV) or Venezuelan equine encephalitis virus (VEEV), wherein each of E2 is bound to each of E3, and an antigen selected from the group consisting of an antigen derived from Plasmodium falciparum circumsporozoite protein, an antigen derived from PD-1, an antigen derived from a ligand of PD-1 (e.g. PD-L1, PD-L2), an antigen derived from CTLA-4, an antigen derived from IL-2, an antigen derived from DISC1, an antigen derived from HER2, an antigen derived from BTLA, an antigen derived from HVEM, an antigen derived from PCSK9 and an antigen derived from DPP-4 is inserted into E3 of Chikungunya virus (CHIKV) or Venezuelan equine encephalitis virus (VEEV). For example, present application provides a virus like particle comprising or consisting of:

240 capsids of Chikungunya virus (CHIKV) or Venezuelan equine encephalitis virus (VEEV);

240 E1s of Chikungunya virus (CHIKV) or Venezuelan equine encephalitis virus (VEEV); and

240 E2s of Chikungunya virus (CHIKV) or Venezuelan equine encephalitis virus (VEEV);

240s E3s of Chikungunya virus (CHIKV) or Venezuelan equine encephalitis virus

(VEEV), wherein each of E2 is bound to each of E3, and an antigen selected from the group consisting of an antigen derived from Plasmodium falciparum circumsporozoite protein, PD-1, an antigen derived from a ligand of PD-1 (e.g. PD-L1, PD-L2), an antigen derived from CTLA-4, an antigen derived from IL-2, an antigen derived from DISC1, an antigen derived from HER2, an antigen derived from BTLA an antigen derived from HVEM, an antigen derived from PCSK9 and antigen derived from DPP-4 is inserted into each of E3s of Chikungunya virus (CHIKV) or Venezuelan equine encephalitis virus (VEEV).

[0086] Virus like particle may work as a carrier of an antigen, which can be administered to human body. Examples of CHIKV VLP, which can work as a carrier of an antigen, include, but are not limited to, Chikungunya virus like particle comprising or consisting of a complex of E2 and E3, capsid and E1, wherein the capsid consists of an amino acid sequence represented by SEQ ID NO: 31(or SEQ ID NO: 75); the E1 consists of an amino acid sequence represented by SEQ ID NO: 32 (or SEQ ID NO: 76); and the complex of E2 and E3 consists of an amino acid sequence represented by SEQ ID NO: 33 (or SEQ ID NO: 77) provided that an amino acid sequence of the at least one antigen is inserted between residues corresponding to 321 and 326 of SEQ ID NO: 1 or SEQ ID NO: 2. Examples of VEEV VLP, which can work as a carrier of an antigen, include, but are not limited to, Venezuelan equine encephalitis virus like particle comprising or consisting of a complex of E2 and E3, capsid and E1, wherein the capsid consists of an amino acid sequence represented by SEQ ID NO: 35; the E1 consists of an amino acid sequence represented by SEQ ID NO: 36; and the complex of E2 and E3 consists of an amino acid sequence represented by SEQ ID NO: 37 provided that an amino acid sequence of the at least one antigen is inserted between residues corresponding to 330 and 335 of SEQ ID NO: 3.

[0087] Examples of a virus like particle comprising Chikungunya viral structural protein and an antigen derived from Plasmodium falciparum circumsporozoite protein, an antigen derived from PD-1, an antigen derived from PD-L1, an antigen derived from CTLA-4, an antigen derived from IL-2, an antigen derived from DISC1, an antigen derived from HER2, an antigen derived from BTLA an antigen derived from HVEM, an antigen derived from PCSK9 and an antigen derived from DPP-4 include, but are not limited to, Chikungunya virus like particle consisting of a complex of E2 and E3 into which the at least one antigen is inserted, capsid and E1, and wherein the at least one antigen is inserted into E3 region, and wherein amino acid sequence of each of the capsid, E1, complex of E2 and E3 into which the at least one antigen is inserted is described below:

(1)

amino acid sequence of E1 is represented by SEQ ID NO: 32;

amino acid sequence of the complex of E2 and E3 into which the malaria antigen is inserted is represented by SEQ ID NO: 46; and
amino acid sequence of capsid is represented by SEQ ID NO: 31;
(2)
amino acid sequence of E1 is represented by SEQ ID NO: 32;
amino acid sequence of the complex of E2 and E3 into which the PD-1 antigen is inserted is represented by SEQ ID NO: 47; and
amino acid sequence of capsid is represented by SEQ ID NO: 31;
(3)
amino acid sequence of E1 is represented by SEQ ID NO: 32;
amino acid sequence of the complex of E2 and E3 into which the PD-L1 antigen is inserted is represented by SEQ ID NO: 48; and
amino acid sequence of capsid is represented by SEQ ID NO: 31; or
(4)
amino acid sequence of E1 is represented by SEQ ID NO: 32;
amino acid sequence of the complex of E2 and E3 into which the CTLA-4 antigen is inserted is represented by SEQ ID NO: 49; and
amino acid sequence of capsid is represented by SEQ ID NO: 31.

[0088] Examples of a virus like particle comprising Venezuelan equine encephalitis viral structural protein and an antigen derived from Plasmodium falciparum circum-sporozoite protein, an antigen derived from PD-1, an antigen derived from PD-L1, an antigen derived from CTLA-4, an antigen derived from IL-2, an antigen derived from DISC1, an antigen derived from HER2, an antigen derived from BTLA, an antigen derived from HVEM an antigen derived from PCSK9 or an antigen derived from DPP-4 include, but are not limited to, Venezuelan equine encephalitis virus like particle consisting of a complex of E2 and E3 into which the at least one antigen is inserted, capsid and E1, and wherein amino acid sequence of each of the capsid, E1, the complex of E2 and E3 into which the at least one antigen is inserted is described below:

(1)
amino acid sequence of E1 is represented by SEQ ID NO: 36;
amino acid sequence of the complex of E2 and E3 into which the malaria antigen is inserted is represented by SEQ ID NO: 50; and
amino acid sequence of capsid is represented by SEQ ID NO: 35;
(2)
amino acid sequence of E1 is represented by SEQ ID NO: 36;
amino acid sequence of the complex of E2 and E3 into which the PD-1 antigen is inserted is represented by SEQ ID NO: 51; and

amino acid sequence of capsid is represented by SEQ ID NO: 35;

(3)

amino acid sequence of E1 is represented by SEQ ID NO: 36;

amino acid sequence of the complex of E2 and E3 into which the PD-L1 antigen is inserted is represented by SEQ ID NO: 52; and

amino acid sequence of capsid is represented by SEQ ID NO: 35; or

(4)

amino acid sequence of E1 is represented by SEQ ID NO: 36;

amino acid sequence of the complex of E2 and E3 into which the CTLA-4 antigen is inserted is represented by SEQ ID NO: 53; and

amino acid sequence of capsid is represented by SEQ ID NO: 35.

[0089] Further, regarding these embodiments, modified capsid of Chikungunya virus (CHIKV) or Venezuelan equine encephalitis virus (VEEV), modified E1 of Chikungunya virus (CHIKV) or Venezuelan equine encephalitis virus (VEEV) and/or modified complex of E2 and E3 of Chikungunya virus (CHIKV) or Venezuelan equine encephalitis virus (VEEV) may be used for the virus like particle. For example, the modified capsid of Chikungunya virus (CHIKV) or Venezuelan equine encephalitis virus (VEEV) may have at least 70%, 75%, 80%, 85%, 90%, 95% or 98% amino acid sequence identity to the amino acid sequence represented by SEQ ID NO: 31 (or SEQ ID NO: 75) or SEQ ID NO: 35; the modified E1 of Chikungunya virus (CHIKV) or Venezuelan equine encephalitis virus (VEEV) may have at least 70%, 75%, 80%, 85%, 90%, 95% or 98% amino acid sequence identity to the amino acid sequence represented by SEQ ID NO: 32 (or SEQ ID NO: 76) or SEQ ID NO: 36; and/or the modified complex of E2 and E3 of Chikungunya virus (CHIKV) or Venezuelan equine encephalitis virus (VEEV) may have at least 70%, 75%, 80%, 85%, 90%, 95% or 98% amino acid sequence identity to the amino acid sequence represented by SEQ ID Nos.:33 (or SEQ ID NO: 77) or SEQ ID Nos.:37. Also, the modified capsid, E1 and/or a complex of E2 and E3 may be a mutant where at most 10% of the amino acids are deleted, substituted, and/or added based on the capsid consisting of an amino acid sequence represented by SEQ ID NO: 31 (or SEQ ID NO: 75) or SEQ ID NO: 35; E1 consisting of an amino acid sequence represented by SEQ ID NO: 32 (or SEQ ID NO: 76) or SEQ ID NO: 36; and/or a complex of E2 and E3 consisting of consisting of an amino acid sequence represented by SEQ ID NO: 33 (or SEQ ID NO: 77) or SEQ ID NO: 37.

[0090] Virus like particle may be prepared by introducing an expression vector comprising a DNA molecule having a nucleotide sequence encoding the virus like particle into a cell (e.g. 293F cell), culturing the cell and recovering the virus like particle from the conditioned medium using ultracentrifugal method.

[0091] (2) Nucleotide, Vector

In a second aspect, the present application provides a nucleic acid molecule comprising or consisting of a nucleotide sequence encoding a virus like particle comprising a modified envelope protein E3.

[0092] A derivative of the above-described nucleic acid molecule which can be prepared by modifying the above-described nucleic acid molecule is also provided by the present application. The derivative may consist of a nucleotide sequence which has a sequence identity of 70%, 80%, 90%, 95% or 98% or more with the nucleotide sequence of the above-described nucleic acid molecule.

[0093] The nucleic acid molecule provided by the present application may be an isolated nucleic acid molecule encoding a virus like particle (e.g. a Chikungunya virus like particle, Venezuelan equine encephalitis virus like particle) which comprises a viral structural protein with a modified envelope protein E3. One embodiment, the virus like particle comprises a viral structural protein and at least one antigen wherein the at least one antigen is inserted into the envelope protein E3 of the viral structural protein.

[0094] One skilled in the art may prepare the nucleic acid molecule provided by the present application described above based on an exemplary nucleotide sequences of Chikungunya or Venezuelan equine viral structural protein that encode capsid and/or envelope represented by SEQ ID Nos.:54-55.

[0095] In one embodiment, a nucleotide sequence encoding an antigen can be inserted into nucleotide sequence encoding E3 of Chikungunya or Venezuelan equine viral structural protein. For example, nucleotide sequence encoding an antigen is inserted between residues 963 and 969 of SEQ ID NO: 54 (for CHIKV) or between residues 990 and 1006 of SEQ ID NO: 55 (for VEEV) to prepare a nucleic acid molecule consisting of a nucleotide sequence encoding a virus like particle comprising an envelope protein E3, wherein the envelope protein E3 is modified to comprise at least one antigen. Examples of the nucleic acid molecule provided by the present application include, but are not limited to, a nucleic acid molecule consisting of a nucleotide sequence represented by any one of SEQ ID NOs.:38-45. A nucleic acid molecule consisting of a nucleotide sequence which has a sequence identity of 70%, 80%, 90%, 95% or 98% or more with the nucleotide sequence represented by any one of SEQ IDs.:38-45 is also provided.

[0096] In one embodiment, the present application provides a vector comprising the nucleic acid molecule as described above, wherein the vector optionally comprises an expression control sequence operably linked to the nucleic acid molecule. A vector used herein may alter a promoter including enhancer sequence, polyadenylation signal, and antibiotic resistance genes. For example, a vector comprising a nucleic acid molecule which consists of a nucleotide sequence represented by SEQ ID NO: 54 or SEQ ID

NO: 55, wherein a nucleotide sequence encoding at least one antigen is inserted between residues 963 and 969 of SEQ ID NO: 54 (for CHIKV VLP) or between residues 990 and 1006 of SEQ ID NO: 55 (for VEEV-VLP); and an expression control sequence operably linked to the nucleic acid molecule is provided.

[0097] Examples of an expression control sequence include, but are not limited to, promoter such as CMV promoter, phage lambda PL promoter, the E. coli lac, phoA and tac promoters, the SV40 early and late promoters, and promoters of retroviral LTRs.

[0098] In this embodiment, the vector comprising an expression control sequence operably linked to the nucleic acid molecule as described above can be used as an expression vector for preparing the particle provided by the present application.

[0099] The expression vectors can be prepared by a person skilled in the art based on WO2012/006180, the entire contents of which are incorporated by reference herein.

[0100] Examples of vectors which can be used for expressing a virus like particle comprising a fusion protein of a protein derived from Chikungunya virus (CHIKV) and an antigen include a vector shown in VLP_CHI 0.56 vector (SEQ ID NO: 30).

[0101] Based on the VLP_CHI 0.56 vector (SEQ ID NO: 30), a skilled person can prepare vectors which can be used for expressing a virus like particle comprising a fusion protein of a protein derived from Chikungunya virus (CHIKV) and a desired antigen. For example, when a skilled person prepares CHIKV VLP comprising a malaria antigen (Sggnpnanpnanpnanpnanpnanpnaggs (SEQ ID NO: 56))-inserted E3, based on the VLP_CHI 0.56 vector, a skilled person can prepare a vector as described below (SEQ ID NO: 57) where nucleotide encoding the antigen is underlined.

[0102]

[Chem. 4]

gaattcccattgcatacgttgtatccatatcataatatgtacatttatattggctcatgtccaa
cattaccgccatgttgacattgattattgactagttattaatagtaatcaattacggggtcatt
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[Chem. 5]

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gtgcacgaaccccccgttcagcccgaccgctgcgccttatccggtaactatcgtcttgagtcca
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attaaaaatgaagttttaaatcaatctaaagtatatatgagtaaacttggtctgacagttacca

[Chem. 6]

atgcttaatcagtgaggcacctatctcagcgatctgtctatttcgttcatccatagttgcctga
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tgctgcaaggcgattaagttgggtaacgccagggttttcccagtcacgacgttgtaaaacgacg
gccagtgaattccatggtctcaactttc

[0103] Examples of vectors which can be used for expressing a virus like particle comprising a fusion protein of a protein derived from Venezuelan equine encephalitis virus (VEEV) and an antigen include a vector shown in VLP_VEEV 0.66 vector (SEQ ID NO: 34).

[0104] Based on the VLP_VEEV 0.66 vector (SEQ ID NO: 34), a skilled person can prepare vectors which can be used for expressing a virus like particle comprising a fusion protein of a protein derived from Venezuelan equine encephalitis virus (VEEV) and a desired antigen. For example, when a skilled person prepares VEEV VLP comprising a malaria antigen (SGG-qgpgapqgpgapqgpgapqgpgap-GGS (SEQ ID NO: 58))-inserted E3, based on the VLP_VEEV 0.66 vector, a skilled person can prepare a vector as described below (SEQ ID NO: 59) where nucleotide encoding the antigen is underlined (Chem 7-8).

[0105]

[Chem. 7]

gaattcccattgcatacgttgtatccatatcataatatgtacatttatattggctcatgtccaacattaccg
ccatgttgacattgattattgactagttattaatagtaatcaattacggggtcattagttcatagcccatat
atggagttccgcgttacataacttacggtaaatggcccgcctggctgaccgcccaacgacccccgcccattg
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[Chem. 8]

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aagttgggtaacgccagggttttcccagtcacgacgttgtaaaacgacggccagtgaattccatggtc
tcaactttc

[0106] Representative structure of plasmid vector that is used to express viral structural protein wherein a nucleotide sequence encoding at least one antigen is inserted in its E2 and E3 regions is shown in Figure 2.

[0107] A nucleic acid molecule having at least 70%, 75%, 80%, 85%, 90%, 95% or 98% nucleotide sequence identity to the nucleic acid molecule having a nucleotide sequence

represented by any one of SEQ ID Nos:57 and 59 and a nucleic acid molecule which may be a mutant where at most 10% of the amino acids are deleted, substituted, and/or added based on the nucleic acid molecule having a nucleotide sequence represented by any one of SEQ ID Nos.:57 and 59 are also provided by the present application.

[0108] The VLPs described as above may be prepared by stable cell line. The stable cell line can be prepared by using the above-described vectors and according to conventional procedures. For example, the following procedures may be employed to generate a stable cell line:

1. Transfect cells such as 293F cells are transfected with a VLP expression plasmid containing selection marker such as hygromycin B.

2. Incubate the transfected cells for one day

3. Culture the transfected cells in a selection medium containing such as Hygromycin at 150-200 ug/ml for 1-2 weeks.

4. Choose the cells that can grow and be split at least once in the selection medium.

5. Isolate a single cell and confirm the expression of the VLP in the supernatants by western blotting.

[0109] (3) Pharmaceutical composition, kit

In a third aspect, the present application provides a pharmaceutical composition and a kit comprising a pharmaceutical composition, wherein the pharmaceutical composition comprises (i) a virus like particle comprising an modified envelope protein E3 and/or (ii) a nucleic acid molecule consisting of a nucleotide sequence encoding a virus like particle comprising a modified envelope protein E3.

[0110] In one embodiment, the present application provides a pharmaceutical composition or a kit comprising a pharmaceutical composition, wherein the pharmaceutical composition comprises the Alphavirus or Flavivirus virus like particle (e.g. Chikungunya virus like particle or Venezuelan equine encephalitis virus like particle) as described above or the nucleic acid molecule as described above; and a pharmaceutically acceptable carrier. The amount of the Alphavirus or Flavivirus virus like particle and the amount of the nucleic acid molecule in the composition may be 0.00001-1 w/w% of the pharmaceutical composition.

[0111] Dosage amount of the particle provided by the present application (e.g. CHIKV VLP or VEEV VLP) may be 1-500μg/day.

[0112] As described above, in one embodiment, the antigen contained in the virus like particle used for the pharmaceutical composition provided by the present application may be derived from Plasmodium falciparum circumsporozoite protein, PD-1, PD-L1, CTLA-4, IL-2, DISC1, HER2, BTLA, HVEM, PCSK9 or DPP-4.

[0113] The pharmaceutical composition may further comprise an adjuvant. Examples of adjuvants include, but are not limited to, Ribi solution (Sigma Adjuvant system,

Sigma-Aldrich). The pharmaceutical composition provided by the present application may contain a buffering agent such as dibasic sodium phosphate hydrate, sodium dihydrogen phosphate and sodium chloride; and a preserving agent such as thimerosal. In one embodiment, the pharmaceutical composition is an aqueous solution containing 0.001-1 w/w% of a particle (e.g. CHIKV VLP or VEEV VLP) comprising a viral structural protein and an antigen (e.g. antigen derived from Plasmodium falciparum circumsporozoite protein, PD-1 or PD-L1), 1-10w/w% of buffering agent, 0.01-1w/w% of adjuvant and 0.00001-0.001w/w% of preserving agent.

[0114] A skilled person can prepare the pharmaceutical composition using a conventional technique. For example, a particle (e.g. CHIKV VLP or VEEV VLP) comprising a viral structural protein and an antigen (e.g. antigen derived from Plasmodium falciparum circumsporozoite protein, PD-1, PD-L1, CTLA-4, IL-2, DISC1, HER2, BTLA, HVEM, PCSK9 or DPP-4) is mixed with a buffer solution having physiological pH (e.g. pH 5-9, such as pH7) to prepare the pharmaceutical composition.

[0115] In one embodiment, the pharmaceutical composition is a vaccine or an immunostimulant comprising a particle comprising a viral structural protein with a modified envelope protein E3. In one embodiment, the virus like particle comprises a viral structural protein and an antigen (e.g. antigen derived from Plasmodium falciparum circumsporozoite protein, PD-1, PD-L1, CTLA-4, IL-2, DISC1, HER2, BTLA, HVEM, PCSK9 or DPP-4). For example, the vaccine composition provided by the present application can be used for immunotherapy; treating or preventing cancer; treating or preventing infectious disease; or treating or preventing malaria.

[0116] In one embodiment, the pharmaceutical composition is a DNA vaccine comprising a nucleic acid molecule comprising a nucleotide sequence for expressing a particle which comprises a viral structural protein with a modified envelope protein E3. One embodiment, the virus like particle comprises a viral structural protein and an exogenous antigen (e.g. antigen derived from Plasmodium falciparum circumsporozoite protein, PD-1, PD-L1, CTLA-4, IL-2, DISC1, HER2, BTLA, HVEM, PCSK9 or DPP-4). In one embodiment, the DNA vaccine provided by the present application comprises CpG containing oligonucleotide.

[0117] The pharmaceutical composition provided in the third aspect of the present application can be administered one or more times. When the pharmaceutical composition provided in the third aspect of the present application is administered more than one time, different particles provided in the first aspect of the present application (e.g. CHIKV VLP or VEEV VLP) may be used for each of the administration. In one embodiment, combination of immunization using CHIKV VLP provided in the first aspect of the application and immunization using VEEV VLP provided in the first aspect of the application is employed. For example, CHIKV VLP provided in the first

aspect of the present application may be used for the 1st immunization and VEEV VLP provided in the first aspect of the present application may be used for the 2nd immunization, or VEEV VLP provided in the first aspect of the present application may be used for the 1st immunization and CHIKV VLP provided in the first aspect of the present application may be used for the 2nd immunization.

[0118] A skilled person can determine timing of immunization using the composition or vaccine provided by the present application. For example, a 2nd immunization is performed 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 weeks after a 1st immunization.

In one embodiment, the present application provides a kit comprising

(a) a pharmaceutical composition comprising a particle provided in the first aspect of the present application; and

(b) another pharmaceutical composition comprising a particle provided in the first aspect of the present application,

wherein the particle contained in (a) is a virus like particle which is different from the particle contained in (b). In this embodiment, the particle contained in (a) may be Chikungunya virus like particle and the particle contained in (b) may be Venezuelan equine encephalitis virus like particle.

[0119] In one embodiment, the present application provides a kit comprising

(a) a pharmaceutical composition comprising a particle provided in the first aspect of the present application; and

(b) another pharmaceutical composition comprising a particle provided in the first aspect of the present application,(c) one or more pharmaceutical composition, each of which comprises a particle provided in the first aspect of the present application,

wherein (a) is used for priming immunization and (b) and (c) are used for boosting immunization; and the particle contained in (a) is a virus like particle which is different from the particle contained in (b); and the particle contained in (c) is different from the particle contained in (a) and (b), or the same as the particle contained in (a) or (b).

[0120] The respective pharmaceutical compositions contained in the above-described kit may be administered simultaneously, separately or sequentially.

[0121] The Alphavirus or Flavivirus virus like particle (e.g. Chikungunya virus or Venezuelan equine encephalitis virus) provided in the first aspect of the present application or the nucleic acid molecule provided by the second aspect of the application can be used for the pharmaceutical composition provided in the third aspect of the present application.

[0122] For example, Chikungunya or Venezuelan equine encephalitis virus like particle comprising or consisting of:

one or more (e.g. 240) capsid of Chikungunya virus (CHIKV) or Venezuelan equine encephalitis virus (VEEV);

one or more (e.g. 240) E1 of Chikungunya virus (CHIKV) or Venezuelan equine encephalitis virus (VEEV);
one or more (e.g. 240) E2 of Chikungunya virus (CHIKV) or Venezuelan equine encephalitis virus (VEEV); and
one or more (e.g. 240) E3 of Chikungunya virus (CHIKV) or Venezuelan equine encephalitis virus (VEEV), wherein each of E2 is bound to each of E3, and an antigen is inserted into E3 of Chikungunya virus (CHIKV) or Venezuelan equine encephalitis virus (VEEV) may be used for preparing the composition or vaccine provided in the third aspect of the present application. The complex of E2 and E3 into which the antigen is inserted may consist of an amino acid sequence represented by any one of SEQ ID Nos.:46-49; the E1 may consist of an amino acid sequence represented by SEQ ID NO: 32 (or SEQ ID NO: 76); and the capsid may consist of an amino acid sequence represented by SEQ ID NO: 31 (or SEQ ID NO: 75); or
the complex of E2 and E3 into which the antigen is inserted may consist of an amino acid sequence represented by any one of SEQ ID Nos.:50-53; the E1 may consist of an amino acid sequence represented by SEQ ID NO: 35; and the capsid may consist of an amino acid sequence represented by SEQ ID NO: 36.

[0123] (4) Method of producing a virus like particle

In a fourth aspect, the present application provides a method of producing a virus like particle comprising a modified envelope protein E3 comprising:

culturing a cell which is transfected with a vector to express the virus like particle; and purifying the particle.

[0124] The virus like particle provided by the first aspect of the present application can be produced by the method provided by a fourth aspect of the present application.

[0125] In one embodiment, antigen may be an antigen derived from Plasmodium falciparum circumsporozoite protein, an antigen derived from PD-1, an antigen derived from PD-L1 or an antigen derived from CTLA-4, an antigen derived from IL-2, an antigen derived from DISC1, an antigen derived from HER2, an antigen derived from BTLA, an antigen derived from HVEM, an antigen derived from PCSK9 or an antigen derived from DPP-4.

[0126] Various host-vector systems may be used for expression of the virus like particle. Eukaryotic cells can be used for the method provided by the fourth aspect of the present application. Examples of eukaryotic cells include, but are not limited to, insect cells (e.g. sf9 cells, H5 cells), yeast cells (e.g. S. cerevisiae) and mammalian cells (e.g. CHO cells, human embryonic kidney (HEK) 293F cells). Vector used for the method provided by the fourth aspect of the present application comprises a nucleic acid molecule encoding the virus like particle to be expressed. Cells may be transfected with the vector using conventional methods (e.g. lipofection, electroporation). A

skilled person can select culture medium or with DNA methyl transferase inhibitors and histone deacetylase inhibitors such as sodium butyrate, depending on cells used for the method provided by the fourth aspect of the present application. After the transfection, virus like particle can be produced in the cells and/or culture supernatant. Virus like particle may be recovered from the culture supernatant and purified using ultracentrifugation.

[0127] For example, cells are transfected with a vector contains the genes coding for CHIKV structural proteins capsid, 6K, E1, E2 and E3 wherein E3 is modified to contain a desired antigen. The cells transfected with the expression vector produce the proteins and the proteins spontaneously assembled to form the VLPs that can be recovered from the culture medium. The 6K protein acts as a signal sequence for transporting E1 protein to the endoplasmic reticulum, where it is processed by host signal peptidase and it is not assembled into virus particles.

[0128] (5) Method of enhancing the production of a virus like particle

In a fifth aspect, the present application provides a method of enhancing the production of a virus like particle comprising a modified envelop protein E3. In one embodiment, the virus like particle comprises viral structural protein and at least one antigen, comprising

(1) inserting the at least one antigen into an envelope protein E3 of the viral structural protein, and

(2) isolating the virus like particle wherein at least one antigen is inserted into the envelope protein E3 of the viral structural protein.

[0129] At least one antigen may be inserted into a suitable position of E3. In one preferred embodiment, at least one antigen is inserted into furin cleavage site which is present in E3 of viral structural protein (e.g. CHIKV structural protein, VEEV structural protein) so that the virus like particle comprising antigen-inserted E3 can be expressed more efficiently when compared to expression of virus like particle comprising E2 into which at least one antigen is inserted.

[0130] For example, regarding Chikungunya viral structural protein, at least one antigen is inserted between residues H at 321-position and S at 326-position of SEQ ID NO: 1 or 2; between P at 320-position and S at 326-position of SEQ ID NO: 1 or 2; or between S at 319-position and S at 326-position of SEQ ID NO: 1 or 2.

[0131] For example, regarding Venezuelan equine encephalitis viral structural protein, at least one antigen is inserted between G at 330-position and S at 335-position of SEQ ID NO: 3; between P at 329-position and S at 335-position of SEQ ID NO: 3; or between C at 328-position and S at 335-position of SEQ ID NO: 3.

[0132] The step of inserting the at least one antigen into envelope protein E3 of the viral structural protein may be achieved by preparing a nucleic acid molecule comprising a

nucleotide sequence encoding the virus like particle wherein at least one antigen is inserted into the envelope protein E3 of the viral structural protein; and allow the nucleic acid molecule to be expressed using cells such as insect cells (e.g. sf9 cells, H5 cells), yeast cells (e.g. S. cerevisiae) and mammalian cells (e.g. CHO cells, 293F cells).

[0133] The step of isolating the virus like particle wherein at least one antigen is inserted into the envelope protein E3 of the viral structural protein may be achieved by purifying the virus like particle using ultracentrifugation.

[0134] (6) Use of the disclosed virus like particle

In a sixth aspect, the present application provides use of (i) a virus like particle comprising a modified envelope protein E3 and/or (ii) a nucleic acid molecule consisting of a nucleotide sequence encoding a virus like particle comprising a modified envelope protein E3 for the manufacture of a pharmaceutical composition or a kit for treating or preventing cancer, neurological disease, infectious disease, malaria or lifestyle chronic disease; producing an antibody against the at least one antigen in a mammal; modulating an immune response; immunostimulation; inhibiting function of the at least one antigen; or presenting an antigen on macrophage.

[0135] The pharmaceutical composition may be administered to a mammal (e.g. human) intramuscularly (i.m.), intracutaneously (i.c.), subcutaneously (s.c.), intradermally (i.d.) or intraperitoneally (i.p.).

In one embodiment, the pharmaceutical composition is a vaccine, which can be applied to immunotherapy. In one embodiment, when an antigen derived from PD-1, PD-L1, CTLA-4, HER2, BTLA or HVEM is used for manufacturing the virus like particle, the virus like particle may be used for treating cancer or autoimmune disease. In one embodiment, when an antigen derived from PD-1 or PD-L1 is used for manufacturing the virus like particle, the virus like particle may be used for treating or preventing an infectious disease. In one embodiment, when an antigen is derived from Plasmodium falciparum circumsporozoite protein, the virus like particle may be used for treating or preventing malaria. In one embodiment, when an antigen derived from DISC1 is used for manufacturing the virus like particle, the virus like particle may be used for treating or preventing a neurological disease. In one embodiment, when an antigen is derived from PCSK9 or DPP-4, the virus like particle may be used for treating or preventing a lifestyle chronic disease.

[0136] Examples of the cancer which may be treated include, but are not limited to, melanoma, renal cancer, prostate cancer, breast cancer, colon cancer and non-small cell lung cancer. Other examples of the cancer include, but are not limited to, bone cancer, pancreatic cancer, skin cancer, cancer of the head or neck, cutaneous or intraocular malignant melanoma, uterine cancer, ovarian cancer, rectal cancer, cancer of the anal region, stomach cancer, testicular cancer, carcinoma of the fallopian tubes,

carcinoma of the endometrium, carcinoma of the cervix, carcinoma of the vagina, carcinoma of the vulva, Hodgkin's Disease, non-Hodgkin's lymphoma, cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the urethra, cancer of the penis, chronic or acute leukemias including acute myeloid leukemia, chronic myeloid leukemia, acute lymphoblastic leukemia, chronic lymphocytic leukemia, solid tumors of childhood, lymphocytic lymphoma, cancer of bladder, cancer of the kidney or ureter, carcinoma of the renal pelvis, neoplasm of the central nervous system (CNS), primary CNS lymphoma, tumor angiogenesis, spinal axis tumor, brain stem glioma, pituitary adenoma, Kaposi's sarcoma, epidermoid cancer, squamous cell cancer, T-cell lymphoma, environmentally induced cancers including those induced by asbestos, and combinations thereof.

[0137] Examples of infectious disease which may be treated include, but are not limited to, HIV, Influenza, Herpes, Giardia, Malaria, Leishmania, the pathogenic infection by the virus Hepatitis (A, B and C), herpes virus (e.g., VZV, HSV-I, HAV-6, HSV-II, and CMV, Epstein Barr virus), alphavirus, adenovirus, influenza virus, flaviviruses, echovirus, rhinovirus, coxsackie virus, cornovirus, respiratory syncytial virus, mumps virus, rotavirus, measles virus, rubella virus, parvovirus, vaccinia virus, HTLV virus, dengue virus, papillomavirus, molluscum virus, poliovirus, rabies virus, JC virus and arboviral encephalitis virus, pathogenic infection by the bacteria chlamydia, rickettsial bacteria, mycobacteria, staphylococci, streptococci, pneumonococci, meningococci and conococci, klebsiella, proteus, serratia, pseudomonas, legionella, diphtheria, salmonella, bacilli, cholera, tetanus, botulism, anthrax, plague, leptospirosis, and Lymes disease bacteria, pathogenic infection by the fungi Candida (albicans, krusei, glabrata, tropicalis, etc.), Cryptococcus neoformans, Aspergillus (fumigatus, niger, etc.), Genus Mucorales (mucor, absidia, rhizophus), Sporothrix schenkii, Blastomyces dermatitidis, Paracoccidioides brasiliensis, Coccidioides immitis and Histoplasma capsulatum, and pathogenic infection by the parasites Entamoeba histolytica, Balantidium coli, Naegleriafowleri, Acanthamoeba sp., Giardia lambia, Cryptosporidium sp., Pneumocystis carinii, Plasmodium vivax, Babesia microti, Trypanosoma brucei, Trypanosoma cruzi, Leishmania donovani, Toxoplasma gondi, and Nippostrongylus brasiliensis.

[0138] Examples of neurological disease which may be treated include, but are not limited to, Alzheimer disease, Parkinson's disease, epilepsy or Schizophrenia.

[0139] Examples of the lifestyle chronic disease which may be treated, but are not limited to, diabetes or hyperlipidemia.

[0140] When a pharmaceutical composition comprising a viral structural protein and an antigen (e.g. an antigen derived from Plasmodium falciparum circumsporozoite

protein, an antigen derived from PD-1, an antigen derived from PD-L1, an antigen derived from CTLA-4, an antigen derived from IL-2, an antigen derived from DISC1, an antigen derived from HER2, an antigen derived from BTLA, an antigen derived from HVEM, an antigen derived from PCSK9 and an antigen derived from DPP-4) is administered to a mammal (e.g. human), an antibody against the antigen is produced in blood of the mammal. The produced antibody may modulate an immune response; show immunostimulating effects; or inhibit function of the antigen.

[0141] The produced antibody may be humanized using a conventional technique. Using the particle provided in a first aspect of the present application, monoclonal antibody or polyclonal antibody can be prepared. In one embodiment, the present application provides a method for producing an antibody comprising administering the particle provided in a first aspect of the present application to a non-human mammal and humanizing non-human mammal produced antibody.

[0142] As used herein, the term "antibody" refers to molecules which are capable of binding an epitope or antigenic determinant. The term is meant to include whole antibodies and antigen-binding fragments thereof, including single-chain antibodies. Such antibodies include human antigen binding antibody fragments and include, but are not limited to, Fab, Fab' and F(ab')2, Fd, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (sdFv) and fragments comprising either a VL or VH domain. The antibodies can be from any animal origin including birds and mammals. Preferably, the antibodies are mammalian e.g. human, murine, rabbit, goat, guinea pig, camel, horse and the like, or other suitable animals e.g. chicken. As used herein, "human" antibodies include antibodies having the amino acid sequence of a human immunoglobulin and include antibodies isolated from human immunoglobulin libraries or from animals transgenic for one or more human immunoglobulins and that do not express endogenous immunoglobulins, as described, for example, in U.S. Patent No. 5,939,598, the disclosure of which is incorporated herein by reference in its entirety.

[0143] The present application will be described in detail with reference to the following examples, which, however, are not intended to limit the scope of the present application.

Example 1

[0144] EXAMPLE 1: Preparation of Venezuelan equine encephalitis virus (VEEV)-virus like particle (VLP) comprising malaria CSP repeat antigen inserted into envelope protein E3 of the viral structural protein

The following polypeptides of malaria CSP protein were used for preparing a VEEV-VLP comprising malaria CSP repeat antigen. SGG is the N terminal linker and GGS is the C terminal linker for the antigen.

[0145] 74 (6 repeat of NPNA amino acid sequence)

Sggnpnanpnanpnanpnanpnaggs (SEQ ID NO: 56)

(Tccggaggaaacccgaatgccaatcccaacgcgaaccccaatgctaacccaaatgccaacccaaacgccaacccca acgctggtggatcc) (SEQ ID NO: 60)

[0146] 76 (14 repeat of NPNA amino acid sequence)

Sggnpnanpnanpnanpnanpnanpnanpnanpnanpnanpnanpnanpnanpnaggs (SEQ ID NO: 61)

(tccggaggcaaccccaacgccaaccctaatgccaatcccaacgctaatcccaatgctaaccctaacgcaaatccaaatg caaaccccaatgccaacccaaacgctaaccctaacgccaaccctaacgcaaacccaaacgccaatcctaatgctaacccaa atgcaaaccctaatgctggcggatcc) (SEQ ID NO: 62)

[0147] 78 (25 repeat of NPNA amino acid sequence)

Sggnpnanpnanpnanpnanpnanpnvdpnanpnanpnanpnanpnanpnanpnanpnanpnanpnanpnanpn anpnanpnanpnanpnanpnanpnanpnanpnaggs (SEQ ID NO: 63)

(tccggaggaaacccgaatgccaatcccaacgcgaaccccaacgctaaccccaacgccaatccgaatgcaaacccgaa cgttgacccaaacgccaacccgaatgccaatcccaacgcgaaccccaatgctaacccaaatgccaacccaaacgccaacc ccaacgctaatccaaacgccaaccctaacgccaatcccaacgcgaatcctaacgctaatcccaacgcaaatcccaatgctaa tccgaacgcgaaccctaatgcaaaccccaacgccaacccgaacgctaacccgaacgctaatcccaacgccggtggatcc) (SEQ ID NO: 64)

[0148] The respective polynucleotides were inserted between the codons encoding Ser at 518-position and Ser at 519-position of SEQ ID NO: 3 to construct a plasmid (hereinafter referred to as pVEEV-74.21, pVEEV-76.21 and pVEEV-78.21, respectively) for expressing VEEV- VLP where the respective antigen is inserted into E2 of Venezuelan equine encephalitis viral structural protein. Likewise, the respective polynucleotides were inserted between the codons encoding Gly at 330-position and Ser at 335-position of SEQ ID NO: 3 to construct a plasmid (hereinafter referred to as pVEEV-74.26, pVEEV-76.26 and pVEEV-78.26, respectively) for expressing VEEV-VLP where the respective antigen is inserted into E3 of Venezuelan equine encephalitis viral structural protein.

[0149] 293F cells (Lifetechnology) were transfected with 180μg of each of the plasmid (i.e. pVEEV-74.21, pVEEV-76.21, pVEEV-78.2, pVEEV-74.26, pVEEV-76.26 and pVEEV-78.26, respectively) using PEI (GE Healthcare). 4 days after the transfection, the conditioned medium was collected and centrifuged at 3000rpm for 15 minutes to separate it from cells. The supernatant was filtrated using 0.45μm filter to obtain virus like particles. The virus like particles were concentrated using TFF column and purified using QXL column (GE Healthcare) to obtain purified virus like particles. When animals were immunized with virus like particles, the purified virus like particles were further concentrated using spin column (Molecular Weight-cutoff: 100kDa) to prepare the virus like particles for the immunization.

[0150] The production of VLP comprising the CSP repeat antigen 74, 76 or 78 conjugated with VEEV structural polypeptide was confirmed by Western Blot using an anti-VEEV antibody (see Figure 3). As seen in Figure 3, antigen-inserted VLP was more efficiently produced when the antigen was inserted into E3 compared to when the antigen was inserted into E2.

Example 2

[0151] EXAMPLE 2: Preparation of Venezuelan equine encephalitis virus (VEEV)-virus like particle (VLP) comprising mouse malaria CSP repeat antigen inserted into envelope protein E3 of the viral structural protein

The following polypeptides of mouse malaria CSP protein was used for preparing a VEEV-VLP comprising mouse malaria CSP repeat antigen. SGG is the N terminal linker and GGS is the C terminal linker for the antigen.

[0152] 261 (repeat of "qgpgap")

SGG-qgpgapqgpgapqgpgapqgpgap-GGS (SEQ ID NO: 58)

Tccggaggacagggacctggcgctcctcagggaccaggggcaccacagggcccaggcgccccacaggggcctgg ggcccctgggggatcc (SEQ ID NO: 65)

[0153] The polynucleotides was inserted between the codons encoding Ser at 518-position and Ser at 519-position of SEQ ID NO: 3 to construct a plasmid (hereinafter referred to as pVEEV-261.25) for expressing VEEV- VLP where the antigen was inserted into E2 of Venezuelan equine encephalitis viral structural protein. Likewise, the polynucleotide coding for the peptide was inserted between the codons encoding Gly at 330-position and Ser at 335-position of SEQ ID NO: 3 to construct a plasmid (hereinafter referred to as pVEEV-261.66) for expressing VEEV- VLP where the antigen was inserted into E3 of Venezuelan equine encephalitis viral structural protein.

[0154] 293F cells (Lifetechnology) were transfected with 180μg of each of the plasmid (i.e. pVEEV-261.25 and pVEEV-261.66, respectively) using PEI (GE Healthcare). 4 days after the transfection, the conditioned medium was collected and centrifuged at 3000rpm for 15 minutes to separate it from cells. The supernatant was filtrated using 0.45μm filter to obtain virus like particles. The virus like particles were concentrated using TFF column and purified using QXL column (GE Healthcare) to obtain purified virus like particles. When animals were immunized with virus like particles, the purified virus like particles were further concentrated using spin column (Molecular Weight-cutoff: 100kDa) to prepare the virus like particles for the immunization.

[0155] The expression of VLP comprising the CSP repeat antigen 261 conjugated with VEEV structural polypeptide was confirmed by Western Blot using an anti-VEEV antibody (see Figure 4). As seen in Figure 4, antigen-inserted VLP was more efficiently produced when the antigen was inserted into E3 compared to when the

antigen was inserted into E2.

Example 3

[0156] EXAMPLE 3: Preparation of Venezuelan equine encephalitis virus (VEEV)-virus like particle (VLP) comprising malaria antigen inserted into envelope protein E3 of the viral structural protein

The following polypeptides derived from malaria protein were used for preparing a VEEV-VLP comprising malaria CSP repeat antigen. SGG is the N terminal linker (TCCGGAGGA in nuclear sequence)and GGS is the C terminal linker (GGAGGATCC in nuclear sequence)for the antigen.

[0157] 74 (6 repeat of NPNA amino acid sequence)

sggnpnanpnanpnanpnanpnanpnaggs (SEQ ID NO: 56)

(tccggaggaaacccgaatgccaatcccaacgcgaaccccaatgctaacccaaatgccaacccaaacgccaaccccaacgctggtggatcc) (SEQ ID NO: 60)

[0158] 302R (antigen derived from pfs25)

SGG-cikidgnpvsyac-GGS (SEQ ID NO: 66)

tccggagggtgcatcaagatcgacggcaaccccgtgtcctacgcctgcgggggatcc

[0159] 303R (antigen derived from pfs25) (SEQ ID NO: 67)

SGG-cildtsnpvktgvc-GGS (SEQ ID NO: 68)

tccggaggctgcatcctggacaccagcaaccccgtgaaaaccggcgtgtgtggcggatcc (SEQ ID NO: 69)

[0160] The respective polynucleotides were inserted between the codons encoding Ser at 518-position and Ser at 519-position of SEQ ID NO: 3 to construct a plasmid (hereinafter referred to as pVEEV-74.21, pVEEV-302R.21 and pVEEV-303R.21, respectively) for expressing VEEV- VLP where the respective antigen is inserted into E2 of Venezuelan equine encephalitis viral structural protein. Likewise, the respective polynucleotides were inserted between the codons encoding Gly at 330-position and Ser at 335-position of SEQ ID NO: 3 to construct a plasmid (hereinafter referred to as pVEEV-74.66, pVEEV-302R.66 and pVEEV-303R.66, respectively) for expressing VEEV- VLP where the respective antigen is inserted into E3 of Venezuelan equine encephalitis viral structural protein.

[0161] 293F cells (Lifetechnology) were transfected with 180μg of each of the plasmid (i.e. pVEEV-74.21, pVEEV-302R.21, pVEEV-303R.2, pVEEV-74.66, pVEEV-302R.66 and pVEEV-303R.66, respectively) using PEI (GE Healthcare). 4 days after the transfection, the conditioned medium was collected and centrifuged at 3000rpm for 15 minutes to separate it from cells. The supernatant was filtrated using 0.45μm filter to obtain virus like particles. The virus like particles were concentrated using TFF column and purified using QXL column (GE Healthcare) to obtain purified virus like particles. When animals were immunized with virus like particles, the purified virus

like particles were further concentrated using spin column (Molecular Weight-cutoff: 100kDa) to prepare the virus like particles for the immunization.

[0162] The expression of VLP comprising antigen 74, 302R or 303R conjugated with VEEV structural polypeptide was confirmed by Western Blot using an anti-VEEV antibody (see Figure 5). As seen in Figure 5, antigen-inserted VLP was more efficiently produced when the antigen was inserted into E3 compared to when the antigen was inserted into E2.

Example 4

[0163] EXAMPLE 4: Preparation of Chikungunya virus (CHIKV)-virus like particle comprising a viral structural protein and PD-1 antigen or PD-1 ligand antigen

The following polypeptides of PD-1 or PD-L1 were used for preparing a CHIKV-VLP comprising PD-1 antigen or PD-1 ligand antigen. SGG is the N terminal linker (TCCGGAGGA in nuclear sequence)and GGS is the C terminal linker (GGAGGATCC in nuclear sequence)for the antigen.

[0164] 1. 299 (mousePD-L1 sequence): A sequence of a fragment of mouse PD-L1 Domain3S attaching linker, which was used for an antigen:

Nuclear Sequence

tccggaggatgcatcatcagctacggcggagccgactacggaggatcc (SEQ ID NO: 70)

Amino Acid sequence

SGG-ciisyggadyC-GGS (SEQ ID NO: 71)

[0165] 2. 274 (mouse PD-1 sequence): A sequence of a fragment of mouse PD-1 domain2short attaching linker, which was used for an antigen:

Nuclear Sequence

tccggaggaggcgccatcagcctgcaccccaaggccaagatcgaggaatctggaggatcc (SEQ ID NO: 72)

Amino Acid sequence

SGG-gaislhpkakiees-GS (SEQ ID NO: 73)

[0166] The respective polynucleotides was inserted between the codons encoding Ser at 531-position and Asn at 532-position of SEQ ID NO: 2 to construct a plasmid (hereinafter referred to as pCHIKV-299.15 and pCHIKV-274.11, respectively) for expressing Chikungunya viral structural protein where the PD-1-derived peptide or the PD-L1-derived peptide is inserted into E2 of Chikungunya viral structural protein. Likewise, the respective polynucleotides were inserted between the codons encoding H at 321-position and S at 326-position of SEQ ID NO: 2 to construct a plasmid (hereinafter referred to as pCHIKV-299.56 and pCHIKV-274.56, respectively) for expressing Chikungunya viral structural protein where the PD-1-derived peptide or the PD-L1-derived peptide is inserted into E3 of Chikungunya viral structural protein.

[0167] 293F cells (Lifetechnology) were transfected with 180μg of each of the plasmid (i.e.

pCHIKV-274.11, pCHIKV-299.15, pCHIKV-274.56 and pCHIKV-299.56, respectively) using PEI (GE Healthcare). 4 days after the transfection, the conditioned medium was collected and centrifuged at 3000rpm for 15 minutes to separate it from cells. The supernatant was filtrated using 0.45μm filter to obtain virus like particles. The virus like particles were concentrated using TFF column and purified using QXL column (GE Healthcare) to obtain purified virus like particles. When animals were immunized with virus like particles, the purified virus like particles were further concentrated using spin column (Molecular Weight-cutoff: 100kDa) to prepare the virus like particles for the immunization.

[0168] The expression of VLP comprising antigen 274 or 299 conjugated with CHIKV structural polypeptide was confirmed by Western Blot using an anti-CHIKV antibody (see Figure 6). As seen in Figure 6, antigen-inserted VLP was more efficiently produced when the antigen was inserted into E3 compared to when the antigen was inserted into E2.

Example 5

[0169] EXAMPLE 5: Preparation of Chikungunya virus (CHIKV)-virus like particle comprising a viral structural protein and malaria antigen

The following polypeptides of malaria CSP protein was used for preparing a CHIKV-VLP comprising malaria CSP repeat antigen. SGG is the N terminal linker (TCCGGAGGA in nuclear sequence)and GGS is the C terminal linker (GGAGGATCC in nuclear sequence)for the antigen.

[0170] CSP repeat antigen 74 (6 repeat of NPNA amino acid sequence)
sggnpnanpnanpnanpnanpnanpnaggs (SEQ ID NO: 56)
(tccggaggaaacccgaatgccaatcccaacgcgaaccccaatgctaacccaaatgccaacccaaacgccaaccccaacgctggtggatcc) (SEQ ID NO: 60)

[0171] The polynucleotide was inserted between the codons encoding Ser at 531-position and Asn at 532-position of SEQ ID NO: 2 to construct a plasmid (hereinafter referred to as pCHIKV-74.11) for expressing Chikungunya viral structural protein where the malaria CSP repeat antigen is inserted into E2 of Chikungunya viral structural protein. Likewise, the malaria CSP repeat antigen was inserted between the codons encoding H at 321-position and S at 326-position of SEQ ID NO: 2 to construct a plasmid (hereinafter referred to as pCHIKV-74.16) for expressing Chikungunya viral structural protein where the malaria CSP repeat antigen is inserted into E3 of Chikungunya viral structural protein.

[0172] 293F cells (Lifetechnology) were transfected with 180μg of each of the plasmid (i.e. pCHIKV-74.11 and pCHIKV-74.16, respectively) using PEI (GE Healthcare). 4 days after the transfection, the conditioned medium was collected and centrifuged at

3000rpm for 15 minutes to separate it from cells. The supernatant was filtrated using 0.45μm filter to obtain virus like particles. The virus like particles were concentrated using TFF column and purified using QXL column (GE Healthcare) to obtain purified virus like particles. When animals were immunized with virus like particles, the purified virus like particles were further concentrated using spin column (Molecular Weight-cutoff: 100kDa) to prepare the virus like particles for the immunization.

[0173] The expression of VLP comprising VLP74 conjugated with CHIKV structural polypeptide was confirmed by Western Blot using an anti-CHIKV antibody (see Figure 7). As seen in Figure 7, antigen-inserted VLP was more efficiently produced when the antigen was inserted into E3 compared to when the antigen was inserted into E2.

Example 6

[0174] EXAMPLE 6: Preparation of Chikungunya virus (CHIKV)-virus like particle comprising a viral structural protein and CTLA-4 antigen

The following polypeptides of CTLA-4 protein was used for preparing a CHIKV-VLP comprising CTLA-4 antigen. SGG is the N terminal linker (TCCGGAGGA in nuclear sequence)and GGS is the C terminal linker (GGAGGATCC in nuclear sequence)for the antigen.

[0175] mCTLA4_ver2

sggggkvelmypppyfvgmggggs (SEQ ID NO: 74)

tccggaggcggcggcaaggtggaactcatgtacccaccgccatactttgtgggcatgggcggcggcggatcc (SEQ ID NO: 78)

[0176] mCTLA4_ver4

sggcattfteknvgfldypfcggs (SEQ ID NO: 79)

tccggaggctgtgccacgacattcacagagaagaatacagtgggcttcctagattacccttctgcggcggatcc (SEQ ID NO: 80)

[0177] mCTLA4_ver5 sggattfteknvgfldypfggs (SEQ ID NO: 81)

tccggaggcgccacgacattcacagagaagaatacagtgggcttcctagattacccttcggcggatcc (SEQ ID NO: 82)

[0178] The polynucleotide was inserted between the codons encoding H at 321-position and S at 326-position of SEQ ID NO: 2 to construct a plasmid for expressing Chikungunya viral structural protein where the mCTLA-4 antigen is inserted into E3 of Chikungunya viral structural protein.

[0179] 293F cells (Lifetechnology) were transfected with 180μg of each of the plasmid using PEI (GE Healthcare). 4 days after the transfection, the conditioned medium was collected and centrifuged at 3000rpm for 15 minutes to separate it from cells. The supernatant was filtrated using 0.45μm filter to obtain virus like particles. The virus like

particles were concentrated using TFF column and purified using QXL column (GE Healthcare) to obtain purified virus like particles. When animals were immunized with virus like particles, the purified virus like particles were further concentrated using spin column (Molecular Weight-cutoff: 100kDa) to prepare the virus like particles for the immunization.

[0180] The expression of VLP comprising each of mCTLA-4_ver2, 4 and 5 conjugated with CHIKV structural polypeptide was confirmed by Western Blot using an anti-CHIKV monkey sera.

Example 7

[0181] EXAMPLE 7 Preparation of mature CHIKV VLP

CHIKV-P2 VLP expression plasmid that can express a CHIKV viral structural protein whose furin site RQRR at the end of E3 region of CHIKV is replaced with SGGGS; CHIKV-Xa VLP expression plasmid that can express a CHIKV viral structural protein whose furin site RQRR at the end of E3 region of CHIKV is replaced with Factor Xa recognition motif, IDGR and CHIKV-En VLP expression plasmid that can express a CHIKV viral structural protein whose furin site RQRR at the end of E3 region of CHIKV is replaced with Enterokinase recognition motif, DDDDK were used. The all three plasmid 10ug were transfected into 293F cells and 4 days after the transfection, the supernatants were harvested. 20ul of supernatants were treated with Water (Control), Factor Xa or Enterokinase for overnight. Then VLPs in the supernatants were measured by Western Blotting using serum against CHIKV. Results are shown in Figure 8.

[0182] Immature VLPs were expressed in the supernatant of the cells transfected with the plasmids, CHIKV-P2, CHIKV-Xa and CHIKV-En. The immature VLP generated from the CHIKV-Xa-transfected cells were digested with Factor Xa. The immature VLP leased E3 and it became mature VLP form (Lane 5). The immature VLP generated from the CHIKV-En-transfected cells were digested with Enterokinase. The immature VLP leased E3 and it became mature VLP form (Lane 9).

Example 8

[0183] EXAMPLE 8 Preparation of mature VEEV VLP

VEEV-P2 VLP expression plasmid that can express a VEEV viral structural protein whose furin site RQRR at the end of E3 region of VEEV is replaced with SGGGS; VEEV-IDGR VLP expression plasmid that can express a VEEV viral structural protein whose furin site RQRR at the end of E3 region of VEEV is replaced with Factor Xa recognition motif, IDGR; and VEEV-IEGR plasmid that can express a VEEV viral structural protein whose furin site RQRR at the end of E3 region of VEEV is replaced with another Xa recognition motif, IEGR were used. The all three plasmids 10ug were

transfected into 293F cells and 4 days after the transfection, the supernatants were harvested. 20ul of supernatants were treated with Water (Control), Factor Xa for overnight. Then VLPs in the supernatants were measured by Western Blotting using serum against VEEV. Immature VLP were expressed in the supernatant of the cells transfected with the plasmids, VEEV-P2, VEEV-IDGR and VEEV-IEGR. The immature VLPs generated from the VEEV-IDGR and VEEV-IEGR-transfected cells were digested with Factor Xa. The result is shown in Figure 9. The immature VLPs generated from the VEEV-IDGR and VEEV-IEGR-transfected cells leased E3 and they became the mature VLP forms (Lane 5 and 6).

Example 9

[0184] EXAMPLE 9: Preparation of Chikungunya virus (CHIKV)-virus like particle comprising DISC1_451, 452 or 454 inserted into envelope protein E3 of the viral structural protein

The following polypeptides of DISC1 protein were used for preparing a CHIKV-VLP comprising DISC1 antigen.

[0185] [Table 1]

Name	Amino acid sequence
DISC1_451	SGGLLIQSLQLQEARGELSVEDERQMDDLEGGS(SEQ ID NO: 105)
DISC1_452	SGGEARGELSVEDERQMDDLEGGS (SEQ ID NO: 106)
DISC1_454	SGGEARGELSVEGGS (SEQ ID NO: 107)

[0186] [Table 2]

Name	DNA sequence
DISC1_451	tccggagggctgctgatccagtctctgcagctgcaggaagccagaggc gagctgagcgtggaagatgagcggcagatggacgacctggaaggggga tcc (SEQ ID NO: 117)
DISC1_452	tccggaggggaagccagaggcgagctgagcgtggaagatgagcggcag atggacgacctggaaggggatcc (SEQ ID NO: 118)
DISC1_454	tccggaggggaagccagaggcgagctgagcgtggaaggggatcc (SEQ ID NO: 119)

[0187] SGG is the N terminal linker (TCCGGAGGA in nuclear sequence)and GGS is the C terminal linker (GGAGGATCC in nuclear sequence)for the antigen.

[0188] The respective polynucleotides was inserted between the codons encoding Ser at 531-position and Asn at 532-position of SEQ ID NO: 2 as well as inserted between the codons encoding H at 321-position and S at 326-position of SEQ ID NO: 2 to construct a plasmid for expressing Chikungunya viral structural protein where the DISC1-derived peptide is inserted into both E2 and E3 of Chikungunya viral structural

protein. The respective polynucleotides was inserted between the codons encoding H at 321-position and S at 326-position of SEQ ID NO: 2 to construct a plasmid (hereinafter referred to as pCHIKV-299.56 and pCHIKV-274.56, respectively) for expressing Chikungunya viral structural protein where the DISC1-derived peptide is inserted into E3 of Chikungunya viral structural protein.
The 293F cells were transfected with the indicated DISC1 expressing VLP. 4 days after transfection, the supernatants were harvested.

[0189] The expression of VLP comprising DISC1_451, _452 or _454 conjugated with CHIKV structural polypeptide was confirmed by Western Blot using an anti-CHIKV antibody (see Figure 10). 1st antibody (1:1000 dilution) was anti-serum against Chikungunya and 2nd antibody (1:5000 dilution) was anti-mouse IgG-HRP antibody. As seen in Figure 10, DISC1_451, _452 and _454 -inserted VLPs were produced when the antigen was inserted into E3 and into both E2 and E3 (dual).

Example 10

[0190] EXAMPLE 10: Preparation of Chikungunya virus (CHIKV)-virus like particle comprising IL-2 inserted into envelope protein E3 of the viral structural protein

Human IL-2 mutant was prepared according to the method described in Levin et. al, Nature 484(7395): 529-533, 2012. Mouse IL-2 mutants are F54A (Mott HR et al. J.Mol.Biol. 247, 979-994, 1995) and D34K (Berndt et al. Biochemistry 33, 6571, 1994), the entire contents of those references are herein incorporated by reference.

[0191] The respective polynucleotides were inserted between the codons encoding H at 321-position and S at 326-position of SEQ ID NO: 2 to construct a plasmid (hereinafter referred to as pCHIKV-299.56 and pCHIKV-274.56, respectively) for expressing Chikungunya viral structural protein where the IL-2-derived peptide is inserted into E3 of Chikungunya viral structural protein. According to the same manner described in EXAMPLE 6, Chikungunya virus like particle comprising IL-2 were prepared and purified.

[0192] The expression of VLP comprising IL-2 conjugated with CHIKV structural polypeptide was confirmed by Western Blot using mouse anti CHIKV antibody (see Figure 11 and 12). As seen in Figures 11 and 12, Human IL-2 wild type, human IL-2 mutant, mouse IL-2 wild type and mouse IL-2 mutant-inserted VLPs were produced when those antigens were inserted into E3.

[0193] CHIKV viral structural proteins comprising IL-2 derived peptide in their envelope protein E3 provided as well as expression vectors expressing the viral structural proteins are SEQ ID NOs.: 83-92. Those sequences contains the 6K protein but the 6K protein acts as a signal sequence for transporting E1 protein to the endoplasmic reticulum, where it is processed by host signal peptidase and it is not assembled into

virus particles.

Example 11

[0194] EXAMPLE 11: Protection of mice against malaria (Plasmodium yoelii) by intramuscle injection of CHIKV VLP comprising mouse malaria (Plasmodium yoelii) inserted into both envelope proteins E2 and E3 of the viral structural protein

Chikungunya VLP comprising Malaria Plasmodium yoelii CSP 4X repeat inserted into both envelop proteins E2 and E3 (261.261 CHIKV VLP) and Chikungunya VLP comprising Malaria Plasmodium yoelii CSP 14X repeat inserted into both envelop proteins E2 and E3 (264.264 CHIKV VLP) were prepared and purified in the similar manner as the previous Examples. Amino acid sequences and nucleotide sequences of the repeat antigens with linker used in this example are SEQ ID NOs.: 93-96. CHIKV viral structural proteins containing the antigens in both E2 and E3 and expression vectors for those viral structural proteins are SEQ ID NOs.: 97-100. Those sequences contains the 6K protein but the 6K protein acts as a signal sequence for transporting E1 protein to the endoplasmic reticulum, where it is processed by host signal peptidase and it is not assembled into virus particles.

[0195] The mice (n=10) were immunized with the 261.261 CHIKV VLP or 264.264 CHIKV VLP 2 times at week 0 and 3 (20ug VLP per mouse) by intramuscle injection. VLPs were mixed with alhydrogel adjuvant before the injection. At week 5, the mice immunized with the 261.261 CHIKV VLP, 264.264 CHIKV VLP and CHIKV VLP with no inserted antigen (Control group) were challenged intravenously with 1000 dose of P. yoelii sporozoites.

[0196] Malaria infection was confirmed by PCR. Genomic DNA was purified from the mice blood day 14 after challenge. 18S malaria DNA was amplified by PCR. Figure 13 shows results of the PCR, indicating that among 10 mice immunized with Control VLP, 9 mice were infected with malaria; among 10 mice immunized with Chikungunya VLP comprising Malaria CSP 4X repeat inserted into both envelop proteins E2 and E3 (261.261 VLP), 9 mice were not infected with malaria; and among 10 mice immunized with Chikungunya VLP comprising Malaria CSP 14X repeat inserted into both envelop proteins E2 and E3, 9 mice were not infected with malaria.

Example 12

[0197] EXAMPLE 12: Immunogenicity of Chikungunya virus (CHIKV)-virus like particles comprising the viral structural protein and human malaria CSP repeat epitope: 74.74 (E2 and E3 (dual) insertion), 74(E2 insertion) and 74(E3 insertion)

Chikungunya viral structural protein comprising Malaria CSP 6X repeat antigen (6XNPNA) in its E2 or E3, as well as in both E2 and E3 were prepared in the similar manner as the previous examples. CHIKV viral structural protein comprising CSP 6X

repeat antigen in both E2 and E3 is SEQ ID NOs: 101 and expression vector for the viral structural protein is SEQ ID NO: 102(74.74.58). CHIKV VLPs comprising the CSP 6X repeat antigen were prepared and purified in the same manner as Example 6.

[0198] The mice (n=4 per group) were immunized with 10ug of indicated VLPs. 10 days after immunization, the anti-CSP antibody titer in the serum of the immunized mice was measured by ELISA coated with recombinant CSP.

[0199] Figure 14 shows that E3-inserted as well as E2- and E3-(dual) inserted VLP had higher titer than E2-inserted VLP.

Example 13

[0200] EXAMPLE 13: Preparation of Chikungunya virus (CHIKV)-virus like particle comprising a viral structural protein and hHER2 antigen

The following polypeptides derived from hHER2 protein were used for preparing a CHIKV-VLP comprising hHER2 antigen. SGG is the N terminal linker (TCCGGAGGA in nuclear sequence)and GGS is the C terminal linker (GGAGGATCC in nuclear sequence)for the antigen.

[0201] [Table 3]

VLP	Amino acid sequence
401	SGGVTYNTDTFESMPGGS (SEQ ID NO: 108)
403	SGGYVNARHCLGGS (SEQ ID NO: 110)
404	SGGYVNARHGLGGS (SEQ ID NO: 111)
405	SGGKFPDEEGACQPCPIGGS (SEQ ID NO: 112)
406	SGGKFPDEEGACQPGGS (SEQ ID NO: 113)
407	SGGKDPPFCVGGS (SEQ ID NO: 114)
408	SGGYKDPPFCVAGGS (SEQ ID NO: 115)
409	SGGYKDPPFCVGGS (SEQ ID NO: 116)

[0202]

[Table 4]

VLP #	DNA sequence
401	aaaaaatccggaggcgtcacctacaacacagacacgtttgagtccatgcccggcg gatccaaa (SEQ ID NO: 120)
403	aaaaaatccggaggctatgtgaatgccaggcactgtttgggcggatccaaa (SEQ ID NO: 121)
404	aaaaaatccggaggctatgtgaatgccaggcacggtttgggcggatccaaa (SEQ ID NO: 122)
405	aaaaaatccggaggcaagtttccagatgaggagggcgcatgccagccttgcccca tcggcggatccaaa (SEQ ID NO: 123)
406	aaaaaatccggaggcaagtttccagatgaggagggcgcatgccagcctggcggat ccaaa (SEQ ID NO: 124)
407	aaaaaatccggaggcaaggaccctcccttctgcgtgggcggatccaaa (SEQ ID NO: 125)
408	aaaaaatccggaggctataaggaccctcccttctgcgtggcgggcggatccaaa (SEQ ID NO: 126)
409	aaaaaatccggaggctataaggaccctcccttctgcgtgggcggatccaaa (SEQ ID NO: 127)

[0203] The respective polynucleotides was inserted between the codons encoding Ser at 531-position and Asn at 532-position of SEQ ID NO: 2 to construct a plasmid for expressing Chikungunya viral structural protein where the hHER2-derived peptide is inserted into E2 of Chikungunya viral structural protein. Likewise, the respective polynucleotides were inserted between the codons encoding H at 321-position and S at 326-position of SEQ ID NO: 2 to construct a plasmid for expressing Chikungunya viral structural protein where the hHER2-derived peptide is inserted into E3 of Chikungunya viral structural protein.

[0204] According to the same manner described in EXAMPLE 6, the Chikungunya virus like particle comprising hHER2 was prepared and purified.

[0205] The expression of VLP comprising hHER2 conjugated with CHIKV structural polypeptide was confirmed by Western Blot using mouse anti CHIKV antibody (see Figure 15). Among them, 401, 404, 406, and 409 were relatively high expression levels and because they code different epitopes.

Example 14

[0206] EXAMPLE 14: Immunogenicity

The mice (n=4 per group) were immunized with 10ug of CHIKV VLP comprising hHER2 antigen #401 inserted into E2 or into both E2 and E3 (dual). The mice (n=10) were immunized with the VLP comprising the hHER2 antigen or CHIKV VLP

comprising no antigen 2 times at week 0 and 3 (20ug VLP per mouse) by intramuscle injection. VLPs were mixed with alhydrogel adjuvant before the injection.

[0207] 2 weeks after 2nd immunization (5 weeks after the 1st immunization), the anti-hHER2 antibody titer in the serum of the immunized mice were measured by ELISA plate coated with recombinant hHER2.

[0208] Figure 16 shows that CHIKV VLP comprising hHER2 antigen #401 in E2 or in both E2 and E3 (dual) generated antibody against hHER2 protein, and dual inserted VLP, i.e. antigen was inserted in both E2- and E3-, could provide higher titer than VLP whose antigen was inserted into E2.

Example 15

[0209] EXAMPLE 15: Preparation of Chikungunya virus (CHIKV)-virus like particle comprising a viral structural protein and hHER2 antigen

The following polypeptides derived from hHER2 protein (GENBANK number: NM_001005862) and derivatives were used for preparing a CHIKV-VLP comprising hHER2 antigen.

[0210] [Table 5]

404(hHer2 original):	sgg	yvnarhgl	ggs
404-1 :	sggcgyvnarhglgcggs		

409 (hHer2 original):	sgg	ykdppfcv	ggs
(409-1):	sgg	ykdppfgv	ggs

[0211] Those amino acid sequences are corresponding to SEQ ID NOs. 128, 129, 130 and 131.

[0212] SGG is the N terminal linker and GGS is the C terminal linker for the antigen.

[0213] The respective polynucleotides were inserted between the codons encoding H at 321-position and S at 326-position of SEQ ID NO: 2 to construct a plasmid for expressing Chikungunya viral structural protein where the hHER2-derived peptide is inserted into E3 of Chikungunya viral structural protein.

[0214] The respective polynucleotides was inserted between the codons encoding Ser at 531-position and Asn at 532-position of SEQ ID NO: 2 to construct a plasmid for expressing Chikungunya viral structural protein where the hHER2-derived peptide is inserted into E2 of Chikungunya viral structural protein.

[0215] Likewise, the respective polynucleotides were inserted both E2 and E3 of chkungunya viral structural protein.

[0216] According to the same manner described in EXAMPLE 6, the Chikungunya virus like particle comprising hHER2 was prepared and purified. The expression of VLP comprising hHER2 that was inserted in E2, E3 or dual (both E2 and E3) of the CHIKV viral structural protein was confirmed by Western Blot using anti CHIKV antibody.

Example 16

[0217] EXAMPLE 16: Preparation of Chikungunya virus (CHIKV)-virus like particle comprising a viral structural protein and BTLA antigen

[0218] The polypeptide of hBTLA (383: SGGCKLNGTTCGGS (SEQ ID NO: 132), derived from GENBANK number: NM_001085357) was used for preparing a CHIKV-VLP comprising hBTLA antigen. SGG is the N terminal linker and GGS is the C terminal linker for the antigen.

[0219] The polynucleotide coding for the peptide was inserted between the codons encoding H at 321-position and S at 326-position of SEQ ID NO: 2 to construct a plasmid for expressing Chikungunya viral structural protein where the hBTLA-derived peptide is inserted into E3 of the Chikungunya viral structural protein.

[0220] According to the same manner described in EXAMPLE 6, the Chikungunya virus like particle comprising hBTLA antigen was prepared and purified.

[0221] Monoclonal antibody was prepared by a conventional procedure from mince immunized with thus obtained VLP. The property of the monoclonal antibody to bind the antigen was measured by ELISA coated with BTLA proteins. Figure 17 shows that CHIKV VLP comprising BTLA antigen could generate monoclonal antibody that binds to both mouse BTLA (mBTLA) and human BTLA (hBTLA) proteins.

Example 17

[0222] EXAMPLE 17: Preparation of Chikungunya (CHIKV)-virus like particle comprising a viral structural protein and HVEM antigen

The following of human HVEM peptides (hHVEM, derived from GENBANK number: NM_001297605) were used for preparing a CHIKV-VLP comprising hHVEM antigen.

354: SGGCVKEASGELTGTVCGGS (SEQ ID NO: 133)

356: SGGCYRVKEASGELTGTVSEPCGGS (SEQ ID NO: 134)

362: SGGCSRNSSRTENAVCGGS (SEQ ID NO: 135)

372: SGGCQMSDPAMGLRSRNCGGS (SEQ ID NO: 136)

[0223] SGG is the N terminal linker and GGS is the C terminal linker for the antigen. The polynucleotide coding for the peptide was inserted between the codons encoding H at 321-position and S at 326-position of SEQ ID NO: 2 to construct a plasmid for expressing Chikungunya viral structural protein where the hHVEM-derived peptide is inserted into E3 of Chikungunya viral structural protein.

[0224] According to the same manner described in EXAMPLE 6, the Chikungunya virus like particle comprising hHHVEM was prepared and purified. The expression of VLP comprising hHVEM conjugated with CHIKV structural polypeptide was confirmed (see Figure 18).

Example 18

[0225] EXAMPLE 18: Expression of immature CHIKV VLP from stable cell line

The transfected cell line that expresses immature VLP obtained in EXAMPLE 8 was prepared.

1. 293F cells were transfected with an expression vector used in Example 8 that contains a selection marker such as hygromycin B.

2. The transfected cells were incubated for one day.

3. The transfected cells were cultured in a selection medium containing Hygromycin at 150-200 ug/ml for 1-2 weeks.

4. The cells that could grow and be split at least once in the selection medium were chosen.

5. A single cell was isolated and cloned. Then, the cells were confirmed to secrete the VLP in the supernatants by western blotting.

[0226] The cloned cells were cultured for 3 months in a medium comprising sodium butyrate, supernatant of the culture was obtained and the immature VLP in the supernatant was confirmed. Result is shown in Figure 19. This data shows that the obtained cell line was stable and could continuously generate immature VLP for long term.

Example 19

[0227] EXAMPLE 19: Protection against tumor by PD-L1 VLP

PD-L1 VLP obtained according to Example 4 was used. The mice (n=10 per group) were injected with PBS (control) or PD-L1 VLP at week 0, 3, 6 and 9.

[0228] The mice were challenged with CT26 cell line (1 x 10^6 cells) at week 8. The tumor sizes were measured.

Figure 20 shows that PD-L1 VLP protects the mice against tumor.

Example 20

[0229] EXAMPLE 20: Preparation of a pharmaceutical composition comprising CHikungunya virus (CHIKV) like particle comprising a viral structural protein and malaria antigen which is inserted both in envelope protein E2 and E3 of the viral structural protein

Chikungunya viral structural protein comprising Malaria CSP 14X repeat antigen 76 (14XNPNA) in only E3 or in both E2 and E3 was expressed in 293F cells in the similar manner according to the previous Examples (CHIKV viral structural protein

containing CSP 14X repeat antigen 76 in E2 and E3 is SEQ ID NO: 103 and expression vector for the viral structural protein is SEQ ID NO: 104). The Chikungunya virus like particle was prepared and purified in the similar manner as the previous Examples. The expression of VLP comprising the CSP repeat antigen 76 conjugated with CHIKV structural polypeptide was confirmed by Western Blot against CHIKV-VLP immunized monkey serum. See Figure 21.

[0230] To prepare a pharmaceutical composition which is a vaccine composition, 80μg of the prepared particles was mixed with 1ml of Sucrose Phosphate Solution, pH 7.2, Endotoxin Free (Teknova, SP buffer).

[0231] Groups of BALB/c mice (n=4) were immunized intramuscularly three times at a 3 weeks' interval with 15 μg of thus obtained VLP, with or without Alhydrogel 2% adjuvant. The sera were collected 2 weeks after the third dose. The serum anti-CSP NANP titer was measured by ELISA using $(NANP)_6$ peptide for coating. The results of this experiment are presented in Figure 22. VLP containing CSP epitope NANP stimulated the production of anti-CSP NANP antibodies and the response was enhanced by the use of Alum.

Claims

[Claim 1] A virus like particle comprising a viral structural protein which comprises modified envelope protein E3.

[Claim 2] The virus like particle according to claim 1, wherein the viral structural protein is derived from alphavirus or flavivirus.

[Claim 3] The virus like particle according to claim 2, wherein the viral structural protein is derived from Chikungunya virus or Venezuelan equine encephalitis virus.

[Claim 4] The virus like particle according to claim 3, wherein the virus like particle is derived from Chikungunya virus strain 37997 or strain OPY-1, or Venezuelan equine encephalitis virus strain TC-83.

[Claim 5] The virus like particle according to claim 1, wherein one or more amino acid residues in the envelope protein E3 are replaced, added and/or deleted in amino acid sequence of the viral structural protein.

[Claim 6] The virus like particle according to claim 5, wherein one or more amino acid residues are replaced, added and/or deleted in amino acid sequence at furin site in the envelope protein E3.

[Claim 7] The virus like particle according to claim 1, wherein the viral structural protein comprises capsid, envelope protein E1, envelope protein E2 and envelope protein E3.

[Claim 8] The virus like particle according to claim 1, wherein an at least one antigen is inserted into the envelope protein E3.

[Claim 9] The virus like particle according to claim 8, wherein the at least one antigen is further inserted into the envelope protein E2.

[Claim 10] The virus like particle according to claim 8, wherein the at least one antigen is inserted between residues corresponding to 321 and 326 of SEQ ID NO: 1, residues 321 and 326 of SEQ ID NO: 2 or residues 330 and 335 of SEQ ID NO: 3.

[Claim 11] The virus like particle according to claim 8, wherein the at least one antigen is derived from Plasmodium falciparum circumsporozoite protein, PD-1, PD-L1, CTLA-4, DISC1, IL-2, HER2, BTLA or HVEM.

[Claim 12] The virus like particle according to claim 11, wherein a peptide selected from (NPNA)n (n=4-30), amino acid sequence represented by SEQ ID Nos.6-9 and 15-29 is inserted into the envelope E3 protein.

[Claim 13] The virus like particle according to claim 1, wherein furin cleavage site located in envelope protein E3 is altered or mutated to prevent the furin site from cleaving.

[Claim 14] An isolated nucleic acid molecule comprising a nucleotide sequence encoding the virus like particle according to any one of claims 1-13.

[Claim 15] A vector comprising the nucleic acid molecule according to claim 14, wherein the vector optionally comprises an expression control sequence operably linked to the nucleic acid molecule.

[Claim 16] A pharmaceutical composition or vaccine composition comprising
(a) the virus like particle according to any one of claims 1-13 and
(b) a pharmaceutically acceptable carrier.

[Claim 17] The composition according to claim 16, which is for treating or preventing cancer, neurological disease, infectious disease or malaria; producing an antibody against the at least one antigen in a mammal; modulating an immune response; immunostimulation; inhibiting function of the at least one antigen; or presenting an antigen on macrophage.

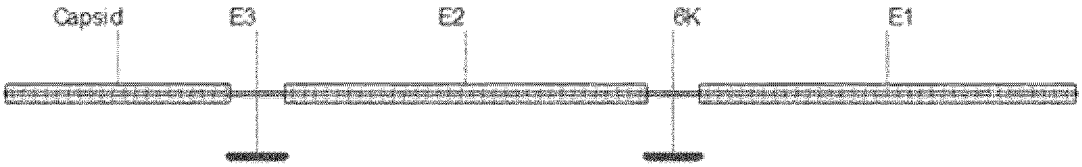
[Claim 18] Use of the virus like particle according to any one of claims 1-13 for the manufacture of a medicament for treating or preventing cancer, neurological disease, infectious disease or malaria; producing an antibody against the at least one antigen in a mammal; modulating an immune response; immunostimulation; inhibiting function of the at least one antigen; or presenting an antigen on macrophage.

[Claim 19] A cell line expressing a viral structural protein which comprises an alternation/mutation to the amino acid sequence at the furin site in the envelope protein E3.

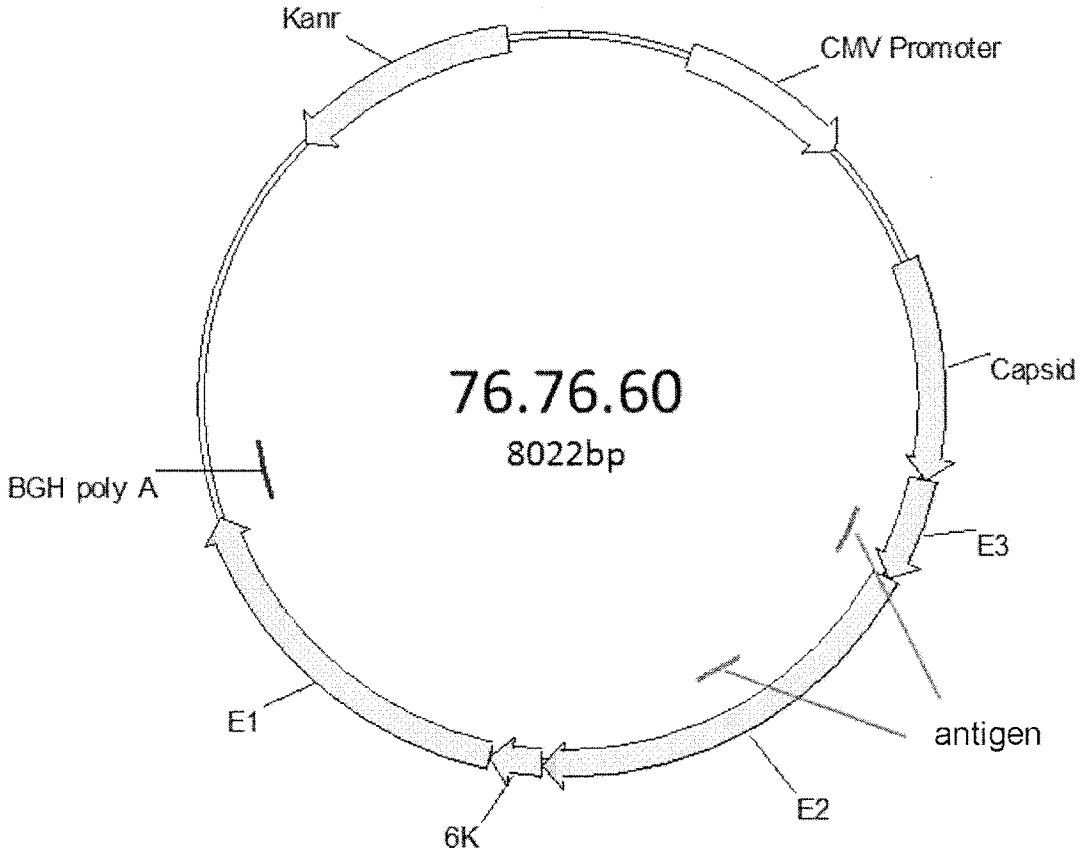
[Claim 20] The cell line according to claim 19, wherein the cell line is a stable cell line.

[Claim 21] The cell line according to claim 19 or 20, wherein the E3 is removed by a protease.

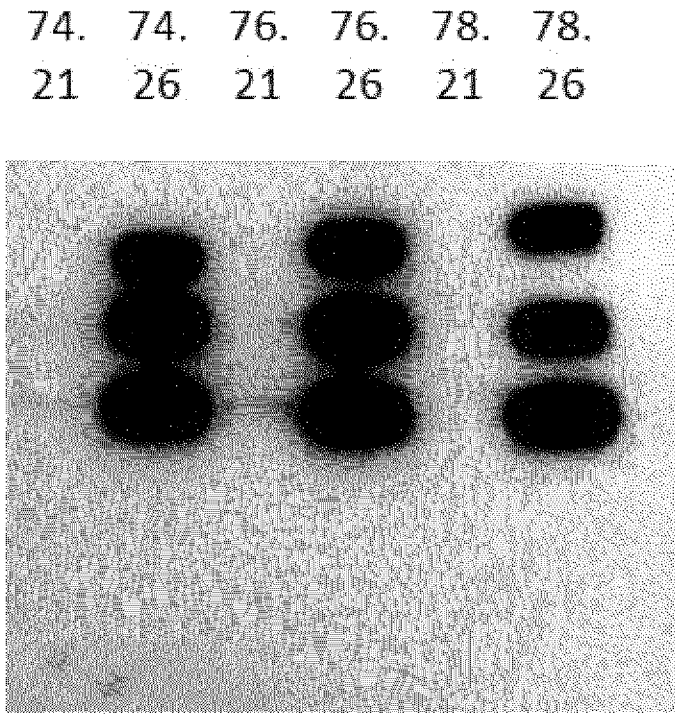
[Fig. 1]


Capsid
E3
E2
6K
E1

[Fig. 2]

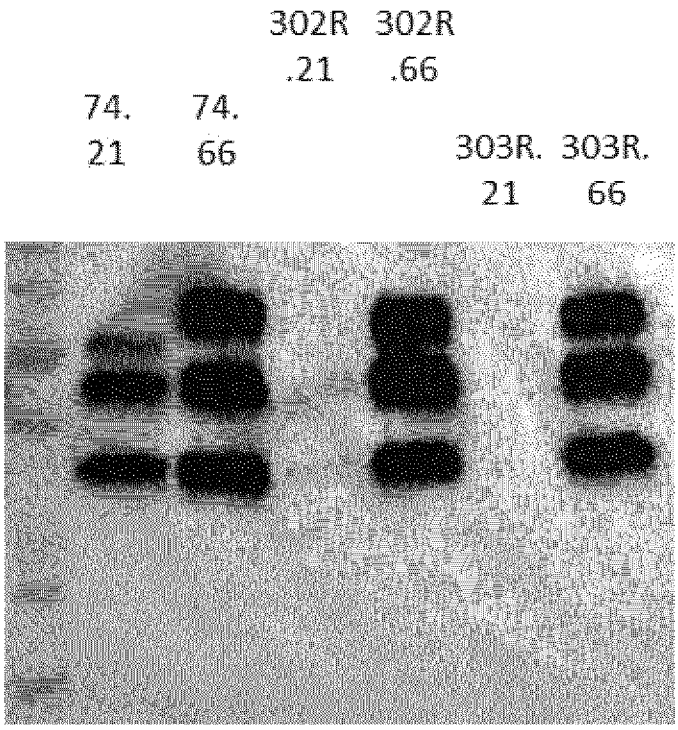

Kanr
CMV Promoter
Capsid
E3
antigen
E2
6K
E1
BGH poly A
76.76.60
8022bp

[Fig. 3]

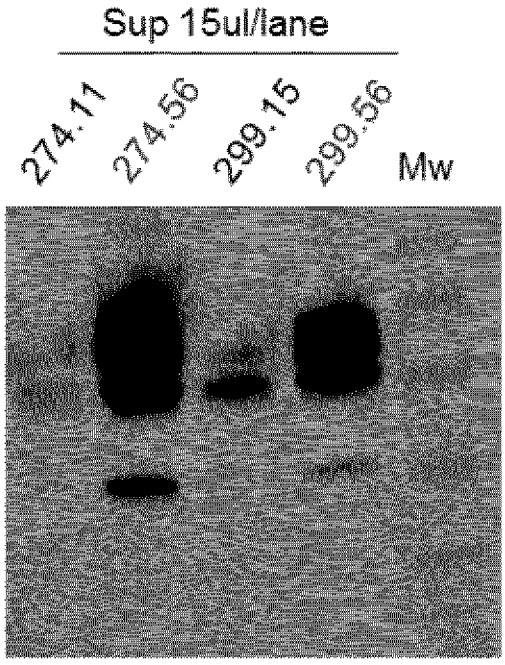


[Fig. 4]

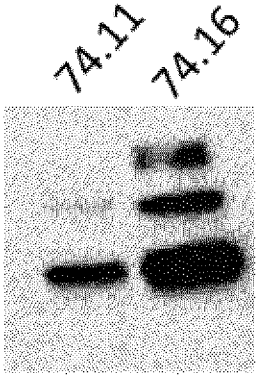
[Fig. 5]



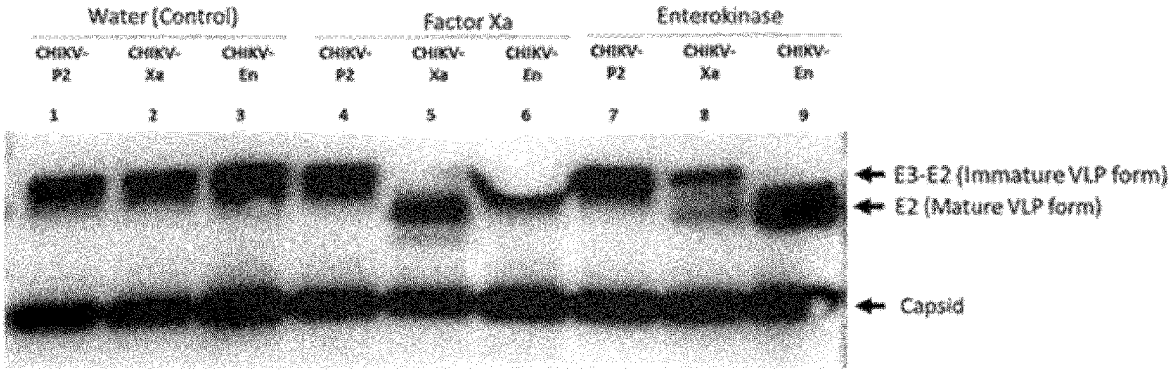
[Fig. 6]



[Fig. 7]

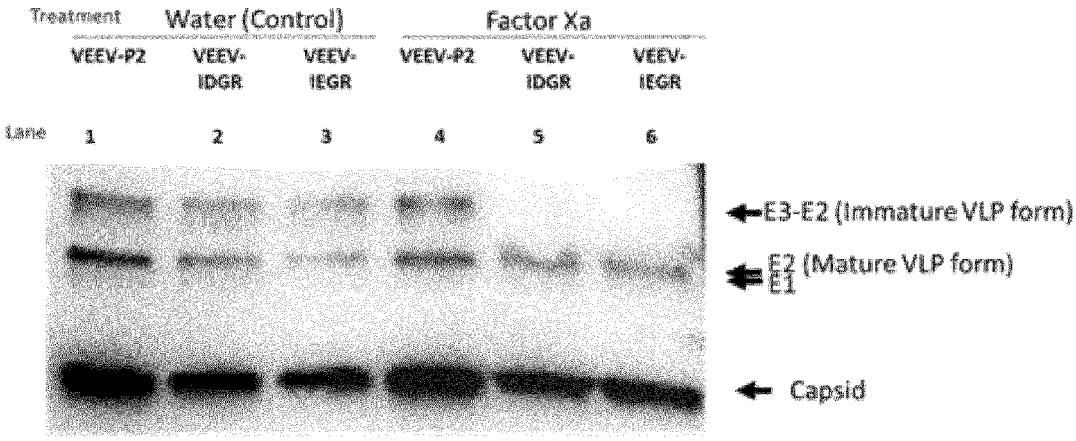


[Fig. 8]



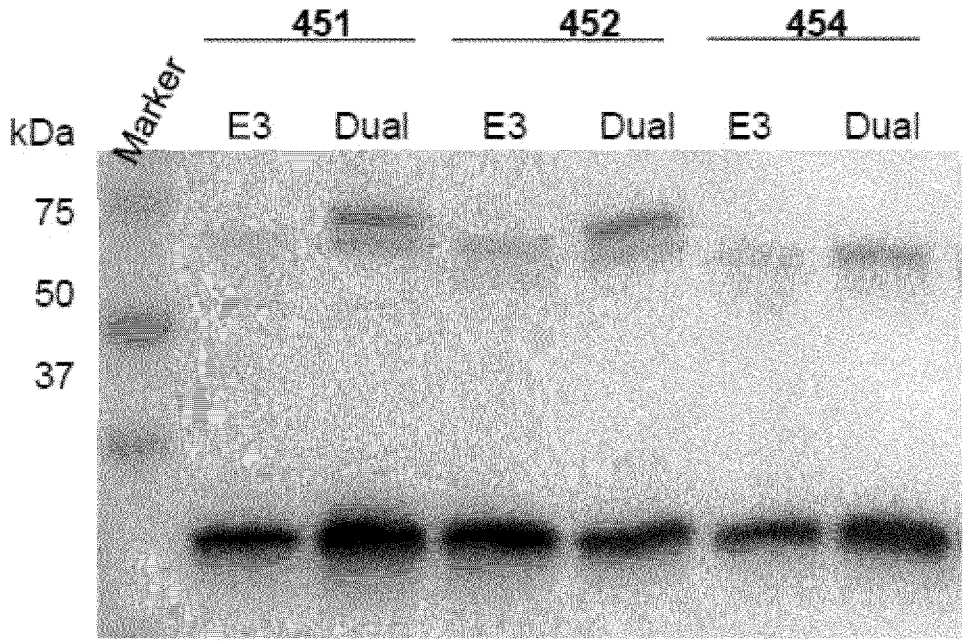
CHIKV- Xa ; Replace furin recognition site to Factor Xa recognition motif (IDGR)
CHIKV- En ; Replace furin recognition site to Enterokinase recognition motif (DDDDK)

[Fig. 9]

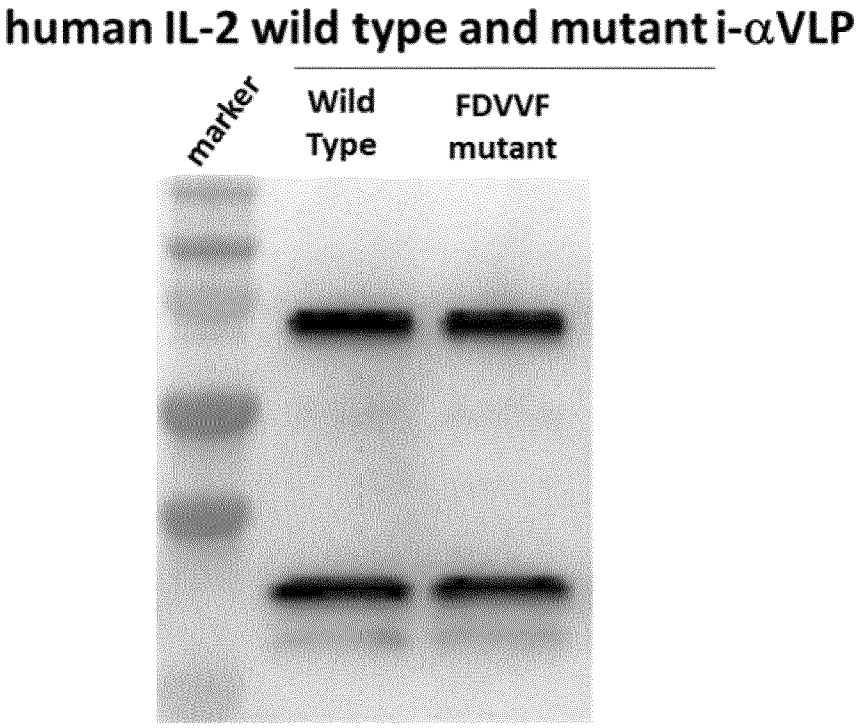


VEEV- IDGR ; Replace furin recognition site to Factor Xa recognition motif (IDGR)
VEEV- IEGR ; Replace furin recognition site to Factor Xa recognition motif (IEGR)

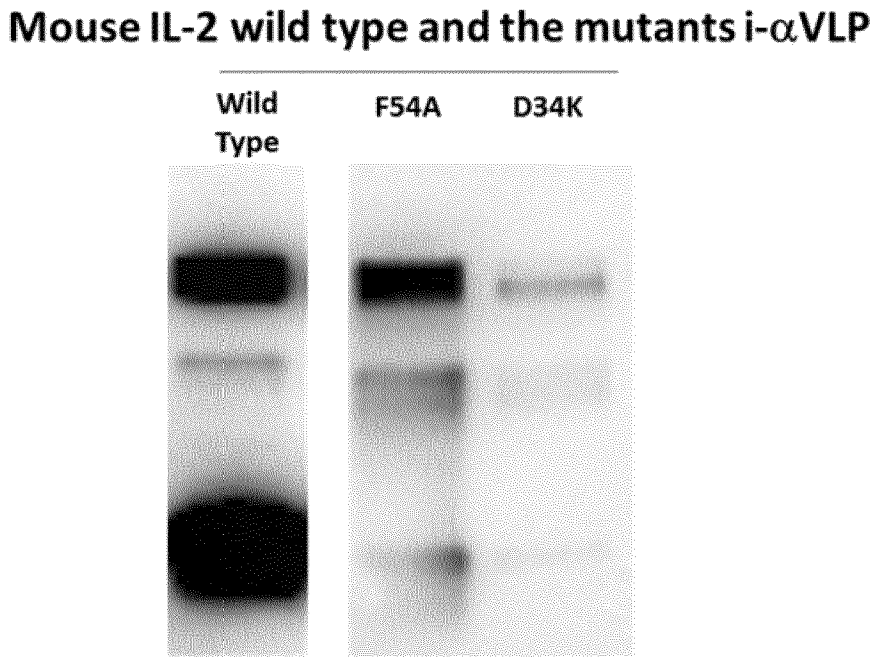
[Fig. 10]



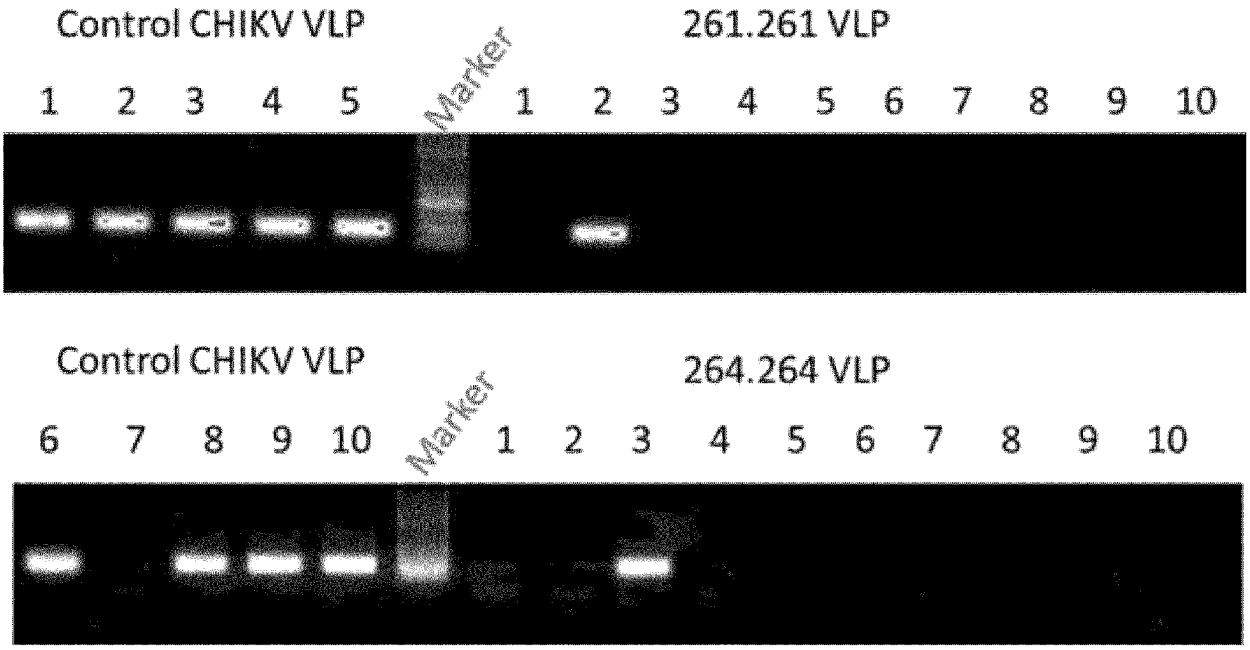
[Fig. 11]


human IL-2 wild type and mutant i-αVLP
marker
Wild Type
FDVVF mutant
Blotting; anti-CHIKV mouse serum 1;1000

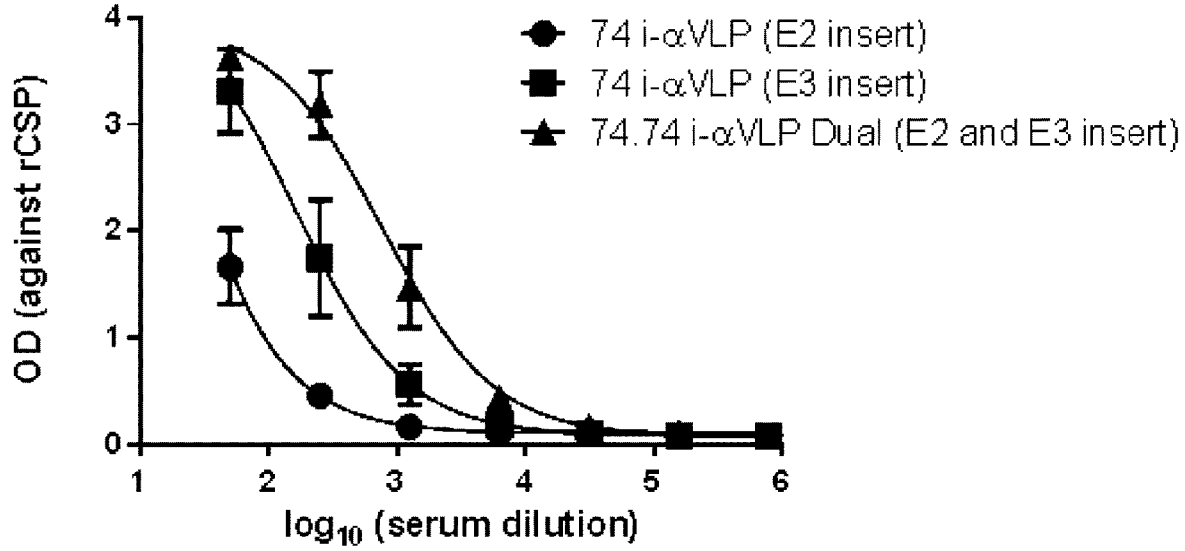
[Fig. 12]


Mouse IL-2 wild type and the mutants i-αVLP
Wild Type
F54A
D34K

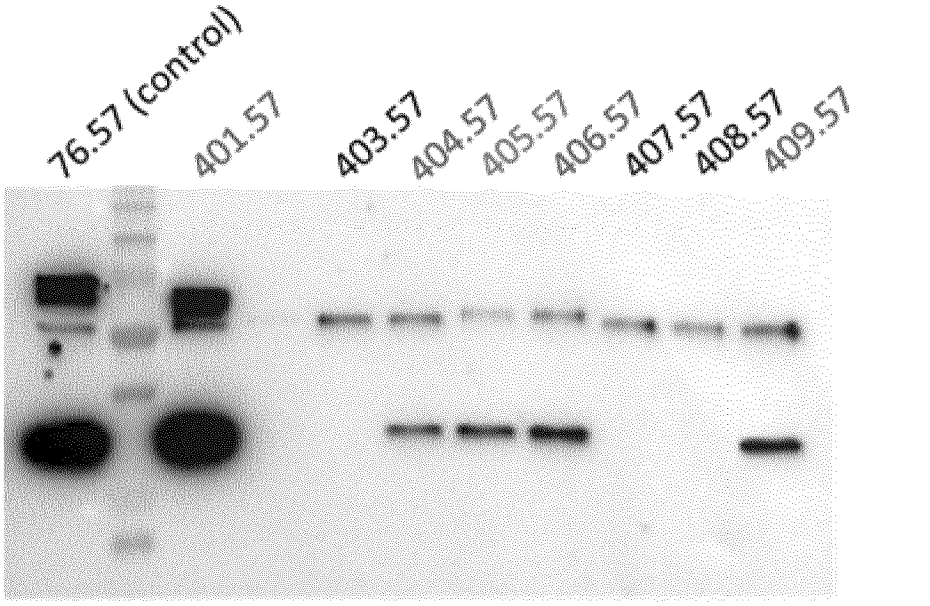
[Fig. 13]


Control CHIKV VLP
261.261 VLP
1 2 3 4 5 Marker 1 2 3 4 5 6 7 8 9 10
Control CHIKV VLP
264.264 VLP
6 7 8 9 10 Marker 1 2 3 4 5 6 7 8 9 10

[Fig. 14]

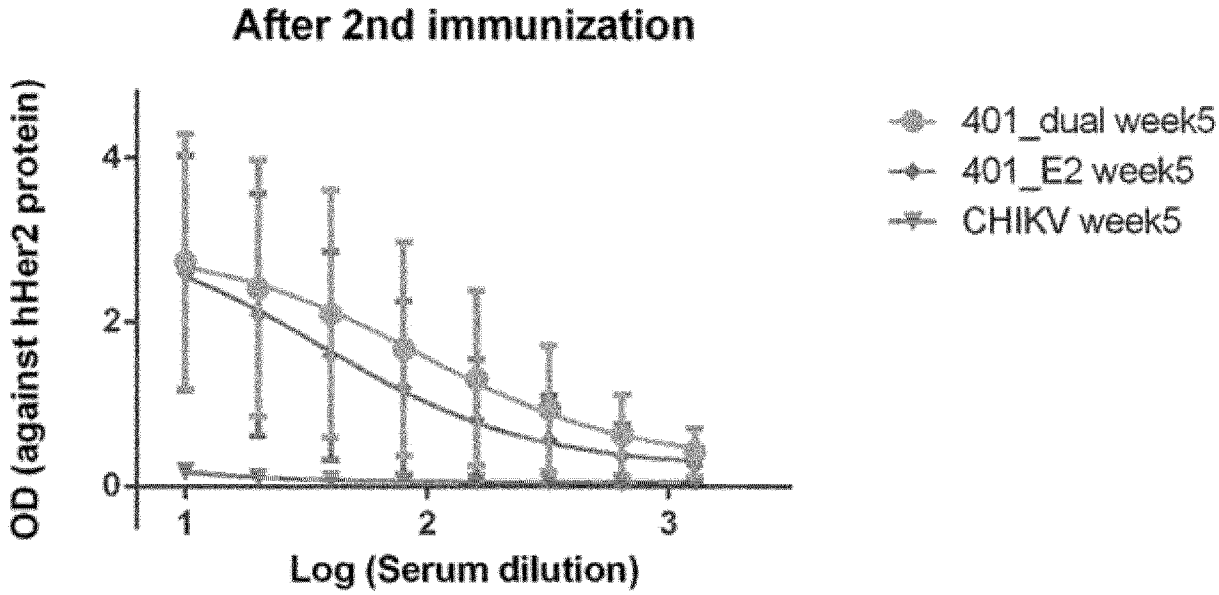

74 i-αVLP (E2 insert)
74 i-αVLP (E3 insert)
74.74 i-αVLP Dual (E2 and E3 insert)
OD (against rCSP)
0
1
2
3
4
1
2
3
4
5
6
log10 (serum dilution)

[Fig. 15]

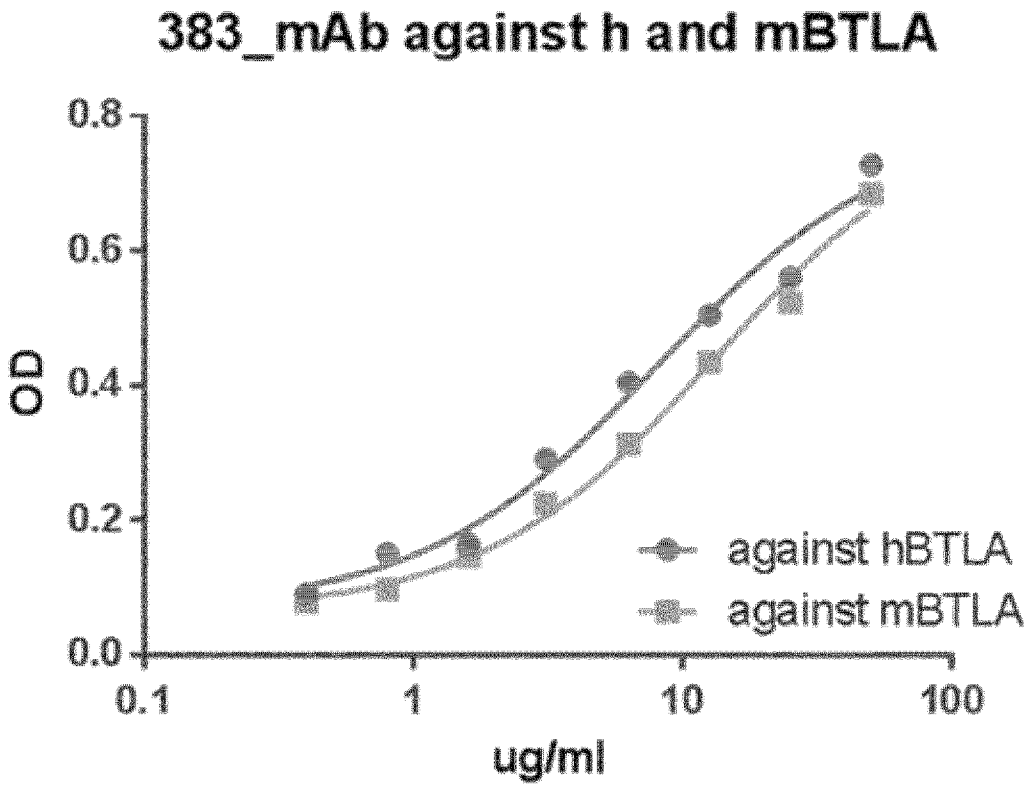


CHIKV VLP immunized mouse serum 1:1000

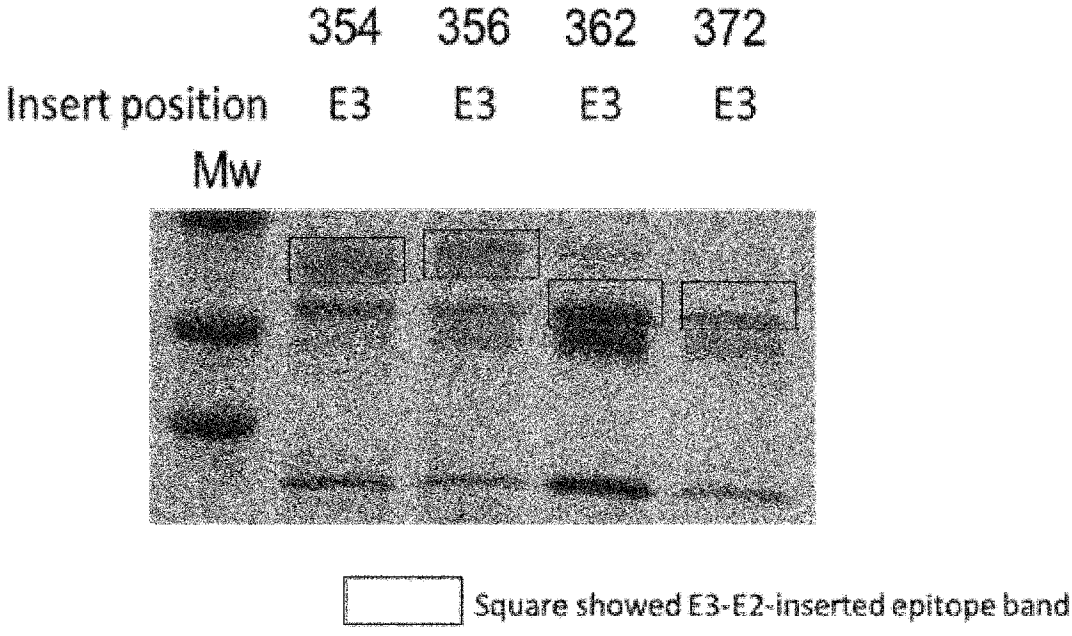
[Fig. 16]



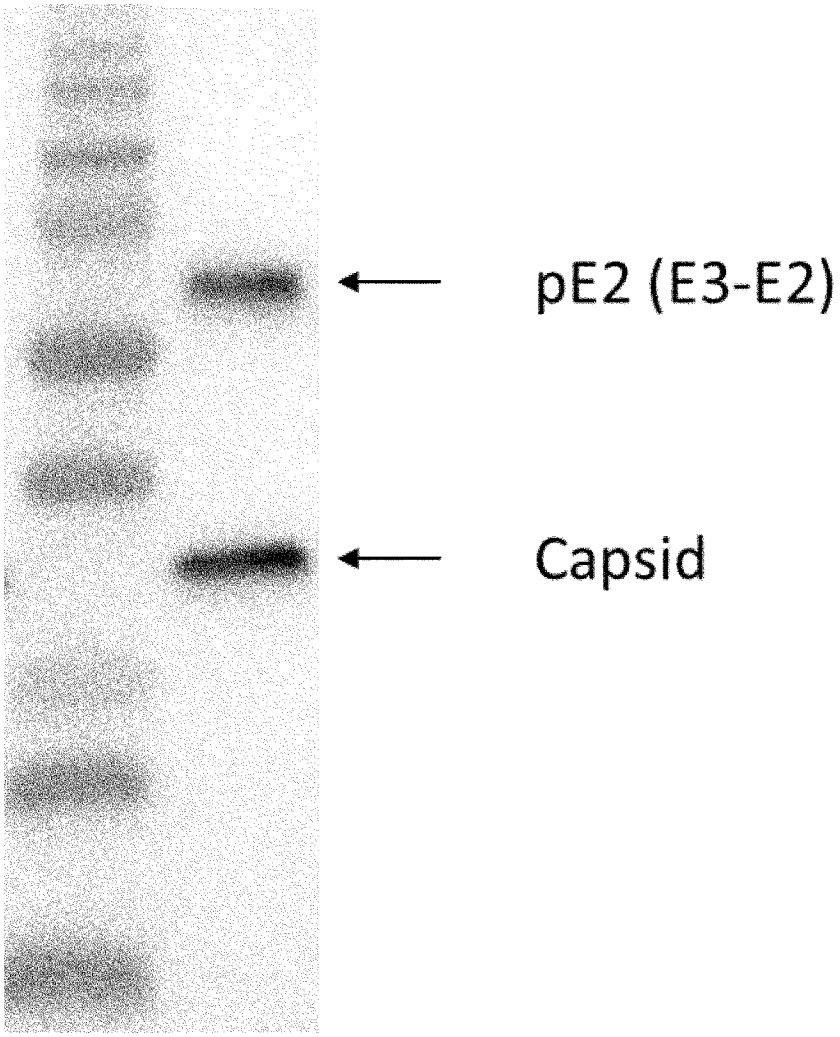
[Fig. 17]


383_mAb against h and mBTLA
0.8
0.6
0.4
0.2
0.0
OD
against hBTLA
against mBTLA
0.1
1
10
100
ug/ml

[Fig. 18]

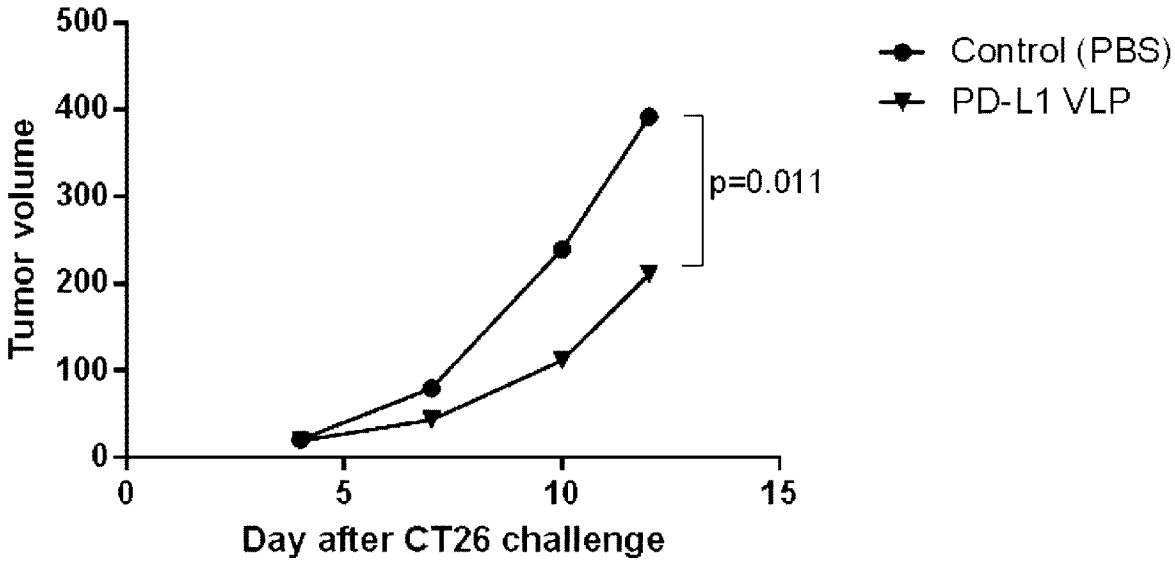

354
356
362
372
Insert position
E3
E3
E3
E3
Mw
Square showed E3-E2-inserted epitope band

[Fig. 19]

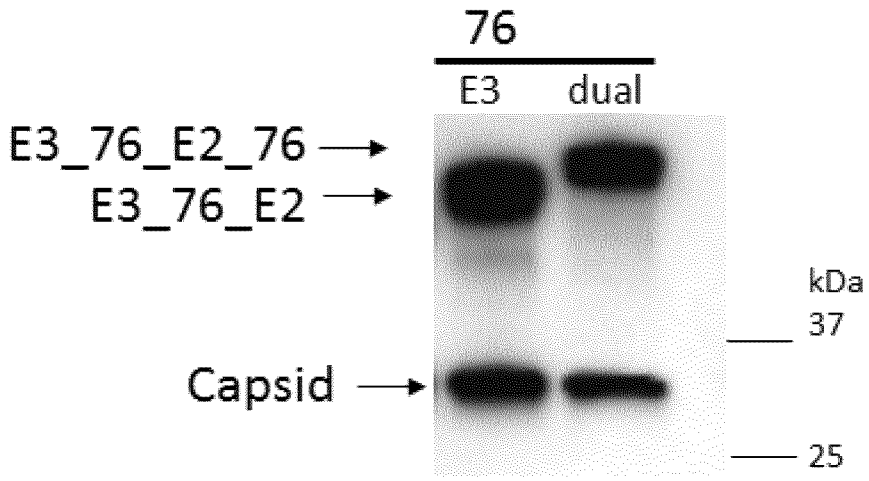


Anti-CHIKV mouse serum blotting (1:1000)

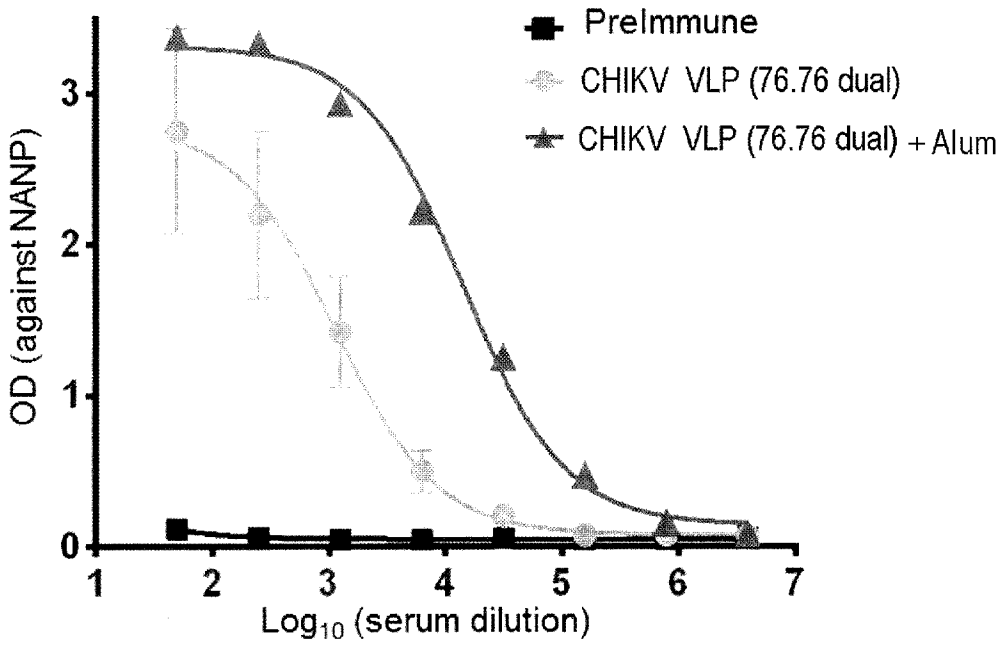
[Fig. 20]



[Fig. 21]


76
E3
dual
E3_76_E2_76
E3_76_E2
Capsid
kDa
37
25

[Fig. 22]


PreImmune
CHIKV VLP (76.76 dual)
CHIKV VLP (76.76 dual) + Alum
OD (against NANP)
0
1
2
3
1
2
3
4
5
6
7
Log10 (serum dilution)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2015/003997

A. CLASSIFICATION OF SUBJECT MATTER

Int.Cl. See extra sheet

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int.Cl. C12N15/09, A61K39/12, A61P31/00, A61P35/00, A61P37/02, C12N5/10, C12N7/00

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

Published examined utility model applications of Japan 1922-1996
Published unexamined utility model applications of Japan 1971-2015
Registered utility model specifications of Japan 1996-2015
Published registered utility model applications of Japan 1994-2015

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

JSTPlus/JMEDPlus/JST580 (JDreamIII), CAplus/MEDLINE/BIOSIS/WPIDS (STN), UniProt/GeneSeq, DWPI (Thomson Innovation)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X/Y/A	WO 2013/122262 A1 (VLP THERAPEUTICS, LLC) 2013.08.22, claims, p.17, lines 1-4, p.22, lines 1-4, p.25, line 20 - p.27, line 17, Table 1 & JP 2015-508648 A, claims, [0026], [0053]-[0055], Table 1 & US 2013/0251744 A1 & EP 2814847 A1	1-5,7-9, 11-12,14-18 /6,19-21 /10,13
Y	GORCHAKOV, R. et al., Comparative analysis of the alphavirus-based vectors expressing Rift Valley fever virus glycoproteins, Virology, 2007, Vol.366, pp.212-225, ISSN 0042-6822, Especially Abstract, p.218, left column lines 18-21, p.218, right column, lines 21-30, Fig.4, Fig.5	6,19-21

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

* Special categories of cited documents:
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13.10.2015	27.10.2015

Name and mailing address of the ISA/JP	Authorized officer	
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2015/003997

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 02/096939 A2 (TRANSGENE S. A.) 2002.12.05, claims, p.27, lines 15-16, p.37, line 11 - p.38, line 7 & JP 2005-500828 A, claims, [0104], [0144]-[0145] & US 2003/0108521 A1 & EP 1390398 A2	1,5,8,14-18
X	WO 2012/172574 A1 (BHARAT BIOTECH INTERNATIONAL LIMITED) 2012.12.20, claims, Table. I & JP 2014-520117 A, claims, Table. I & US 2014/0120125 A1 & EP 2720715 A1	1-3,5,7, 14-18
X	ELSHUBER, S. et al., Cleavage of protein prM is necessary for infection of BHK-21 cells by tick-borne encephalitis virus, Journal of General Virology, 2003, Vol.84, pp.183-191, ISSN 0022-1317, Especially Abstract, pp.185-186 'Mutation of the furin cleavage site in prM', Fig.1, Fig.2	19-21
A	ELSHUBER, S. et al., Resuscitating Mutations in a Furin Cleavage-Deficient Mutant of the Flavivirus Tick-Borne Encephalitis Virus, Journal of Virology, 2005, Vol.79, No.18, pp.11813-11823, ISSN 0022-538X, Especially p.11821, right column, lines 50-52	1-21
A	OZDEN, S. et al., Inhibition of Chikungunya Virus Infection in Cultured Human Muscle Cells by Furin Inhibitors, The Journal of Biological Chemistry, 2008, Vol.283, No.32, pp.21899-21908, ISSN 0021-9258, Especially p.21900, right column, lines 17-19, Fig.1, Fig.3	1-21
P,Y	WO 2015/005500 A1 (VLP THERAPEUTICS, LLC) 2015.01.15, [0029] & US 2015/0017194 A1	11-12, 14-18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2015/003997

CLASSIFICATION OF SUBJECT MATTER
C12N15/09(2006.01)i, A61K39/12(2006.01)i, A61P31/00(2006.01)i,
A61P35/00(2006.01)i, A61P37/02(2006.01)i, C12N5/10(2006.01)i,
C12N7/00(2006.01)i