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- (71) **Applicant:** THEVAX GENETICS VACCINE CO., LTD. [CN/CN]; 3F, No. 25-1, Sec. 4, Ren-ai Rd., Da-an District, Taipei City, Taiwan 10685 (CN).
- (71) **Applicant (for ZA only):** HEALTHBANKS BIOTECH USA INC. [US/US]; 185 Technology Dr., 150, Irvine, CA 92618 (US).
- (72) **Inventors:** WU, Chia-Mao; 2F., No.88, Zhuangjing 1st Rd., Zhubei City, Shinchu County, Taiwan, 302 (CN). CHANG, Hsiu-Kang; 5F., No. 25-1, Sec.4, Ren-ai Rd., Da-an District, Taipei City, Taiwan, 10685 (CN).
- (74) **Agent:** SAUNDERS, Hsiu-Ming; IPC Intellectual Property Connections, Inc., 3346 Ramona Street, Palo Alto, CA 94306 (US).

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(54) **Title:** FUSION PROTEINS FOR USE AS IMMUNOGENIC ENHANCERS FOR INDUCING ANTIGEN-SPECIFIC T CELL RESPONSES

(57) **Abstract:** A fusion protein for use as an immunogen enhancer for enhancing antigen-specific T cell responses is disclosed. The fusion protein comprises: (a) an antigen-presenting cell (APC)-binding domain or a CD91 receptor-binding domain; (b) a protein transduction domain; and (c) an antigen of a pathogen, wherein the APC-binding domain or the CD91 receptor-binding domain is located at the N-terminus of the fusion protein, and the antigen of the pathogen is located at the C-terminus of the protein transduction domain. The protein transduction domain is selected from the group consisting of: (i) a fusion polypeptide, comprising a T cell sensitizing signal-transducing peptide, a linker, and a translocation peptide; (it) a T cell-sensitizing signal-transducing peptide; and (iii) a translocation peptide of 34-112 amino acid residues in length.



FUSION PROTEINS FOR USE AS IMMUNOGENIC ENHANCERS FOR INDUCING ANTIGEN-SPECIFIC T CELL RESPONSES

FIELD OF THE INVENTION

The present invention relates generally to fusion proteins, and more specifically to fusion proteins
5 for enhancing T cell-mediated immune response.

BACKGROUND OF THE INVENTION

Molecular biology has enabled the production of subunit vaccines, in which the immunogen is a
fragment or subunit of a parent protein or complex. The development of a stable vaccine that could elicit
T cell sensitizing responses, and be flexible enough to incorporate sequences from many strains of an
10 infectious agent would be desirable.

SUMMARY OF THE INVENTION

In one aspect, the invention relates to a fusion protein comprising:

- (a) an antigen-presenting cell (APC)-binding domain or a CD91 receptor-binding domain,
located at the N-terminus of the fusion protein;
- 15 (b) a protein transduction domain, located at the C-terminus of the APC-binding domain or the
CD91 receptor-binding domain, the protein transduction domain being selected from the group
consisting of:
 - (i) a fusion polypeptide, comprising a T cell sensitizing signal-transducing peptide, a
linker, and a translocation peptide, wherein:
 - 20 (1) the T cell sensitizing signal-transducing peptide is located at the N-terminus of
the fusion polypeptide;
 - (2) the linker comprises SEQ ID NO: 15, linking the T cell sensitizing signal-
transducing peptide and the translocation peptide; and
 - (3) the translocation peptide has 34-112 amino acid residues in length and comprises
25 the amino acid sequence that is at least 90% identical to SEQ ID NO: 3, 20 or 4;
 - (ii) a T cell-sensitizing signal-transducing peptide; and
 - (iii) a translocation peptide of 34-112 amino acid residues in length, comprising the
amino acid sequence that is at least 90% identical to SEQ ID NO: 3, 20 or 4; and
- (c) an antigen of a pathogen, located at the C-terminus of the protein transduction domain;
- 30 wherein:

the T cell-sensitizing signal-transducing peptide has 28-53 amino acid residues in length and comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 31, in which Xaa⁸ is I or L; Xaa¹⁰ is V, F or A, Xaa¹¹ is M or L, Xaa¹⁷ is L or I; and the APC-binding domain or the CD91 receptor-binding domain is free of the amino acid sequence of Pseudomonas exotoxin A (PE) binding domain I if the protein transduction domain is the translocation peptide (biii).

In one embodiment of the invention, the APC-binding domain or the CD91 receptor-binding domain is a polypeptide comprising an amino acid sequence that is at least 90% identical to the sequence selected from the group consisting of SEQ ID NOs: 5, 9, 6, 7, and 8. Alternatively, the APC-binding domain is selected from the group consisting of receptor-associated protein-1 (RAP1) domain III, alpha-2-macroglobulin receptor-associated protein (A2M), HIV-Tat, and heat shock proteins (HSPs), and Pseudomonas exotoxin A (PE) binding domain I.

In another embodiment of the invention, the fusion protein is free of the amino acid sequence of Pseudomonas exotoxin A (PE) binding domain I.

In another embodiment of the invention, the fusion protein further comprises an endoplasmic reticulum retention sequence located at the C-terminus of the fusion protein.

In another embodiment of the invention, the endoplasmic reticulum retention sequence comprises the amino acid sequence of Lys-Asp-Glu-Leu (SEQ ID NO: 14). The ER retention sequence may comprise a sequence selected from the group consisting of SEQ ID NOs: 14, 16-19. Alternatively, the ER retention sequence may consist of a sequence selected from the group consisting of SEQ ID NOs: 16-19.

In another embodiment of the invention, the fusion protein is free of an endoplasmic reticulum retention sequence at C-terminus thereof if the antigen contains 10 or more epitopes.

In another embodiment of the invention, the protein transduction domain is the fusion polypeptide (bi).

In another embodiment of the invention, the protein transduction domain is the T cell-sensitizing signal-transducing peptide (bii).

In another embodiment of the invention, the fusion protein further comprises an additional linker between the protein transduction domain and the antigen, the additional linker comprising SEQ ID NO:

In another embodiment of the invention, the protein transduction domain is the translocation peptide (biii).

In another embodiment of the invention, the fusion protein further comprises an additional linker between the APC-binding domain or the CD91 receptor-binding domain and the translocation peptide, the additional linker comprising SEQ ID NO: 15.

In another embodiment of the invention, the protein transduction domain comprises the sequence of SEQ ID NO: 30.

In another embodiment of the invention, the APC-binding domain comprises an amino acid sequence that is at least 95% identical to the sequence selected from the group consisting of SEQ ID NOs: 5, 9, 6, 7, and 8.

In another embodiment of the invention, the APC-binding domain or the CD91 receptor-binding domain is a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 5, 9, 6, 7, and 8.

In another embodiment of the invention, the T cell sensitizing signal-transducing peptide comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 1 or 2.

In another embodiment of the invention, the T cell sensitizing signal-transducing peptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 1 and 2.

In another embodiment of the invention, the translocation peptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 3, 20, and 4.

In another embodiment of the invention, the translocation peptide has 34-61 amino acid residues in length.

In another embodiment of the invention, the protein transduction domain of the fusion protein as aforementioned possesses the following features: (i) the T cell-sensitizing signal-transducing peptide comprises the amino acid sequence of SEQ ID NO: 1 or 2; and (ii) the translocation peptide comprises the amino acid sequence that is at least 95% identical to SEQ ID NO: 3.

The T cell sensitizing signal-transducing peptide exhibits a characteristic of eliciting an antibody that recognizes and binds to the amino acid sequence of $K^1(X)^2E^3(X)^4(X)^5Y^6P^7P^8P^9Y^{10}$ (SEQ ID NO: 32) of CD28 receptor on T cells, wherein $(X)^2$ is I or L; $(X)^4$ is V, F or A, and $(X)^5$ is M or L.

In another aspect, the invention relates to a fusion protein consisting of:

- (a) an antigen-presenting cell (APC)-binding domain or a CD91 receptor-binding domain, located at the N-terminus of the fusion protein;

(b) a protein transduction domain, located at the C-terminus of the APC-binding domain or the CD91 receptor-binding domain, the protein transduction domain being selected from the group consisting of:

(i) a fusion polypeptide, comprising a T cell sensitizing signal-transducing peptide, a linker, and a translocation peptide, wherein:

(1) the T cell sensitizing signal-transducing peptide is located at the N-terminus of the fusion polypeptide;

(2) the linker comprises SEQ ID NO: 15, linking the T cell sensitizing signal-transducing peptide and the translocation peptide; and

(3) the translocation peptide has 34-112 amino acid residues in length and comprises the amino acid sequence that is at least 90% identical to SEQ ID NO: 3, 20 or 4;

(ii) a T cell-sensitizing signal-transducing peptide; and

(iii) a translocation peptide of 34-112 amino acid residues in length, comprising the amino acid sequence that is at least 90% identical to SEQ ID NO: 3, 20 or 4; and

(c) an antigen of a pathogen, located at the C-terminus of the protein transduction domain; wherein:

the T cell-sensitizing signal-transducing peptide has 28-53 amino acid residues in length and comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 31, in which Xaa⁸ is I or L; Xaa¹⁰ is V, F or A, Xaa¹¹ is M or L, Xaa¹⁷ is L or I; and

the APC-binding domain or the CD91 receptor-binding domain is free of the amino acid sequence of Pseudomonas exotoxin A (PE) binding domain I if the protein transduction domain is the translocation peptide (biii).

The antigen-presenting cell (APC) may be selected from the group consisting of dendritic cells, macrophages, B-cells and monocytes.

In one embodiment of the invention, the cell membrane of the APC comprises a CD91 receptor.

In another aspect, the invention relates to a vaccine composition comprising: (a) a therapeutically effective amount of a fusion protein as aforementioned; and (b) an adjuvant.

The adjuvant is either an antigen delivery agent or an immune potentiator. In one embodiment of the invention, the vaccine composition comprises an antigen delivery agent and is free of an immune potentiator.

Further in another aspect, the invention relates to a method for inducing enhanced pathogen antigen-specific T cell responses, comprising: administering a vaccine composition comprising a therapeutically effective amount of a fusion protein as aforementioned to a subject in need thereof, and thereby inducing enhanced pathogen antigen-specific T cell responses.

5 Further in another aspect, the invention relates to a method for killing a disease cell that presents an antigen via class I MHC molecules on the cell membrane of the disease cell, comprising: administering a vaccine composition comprising a therapeutically effective amount of a fusion protein as aforementioned to a subject in need thereof, and thereby killing the disease cell that that presents the antigen via class I MHC molecules on the cell membrane of the disease cell.

10 In one embodiment of the invention, the disease cell is a cancer cell.

Yet in another aspect, the invention relates to a method for preventing, treating infection caused by a pathogen, and/or minimizing symptoms caused by the infection, comprising: administering a vaccine composition comprising a therapeutically effective amount of the fusion protein of claim 1 to a subject in need thereof, and thereby preventing, treating infection caused by the pathogen, and/or minimizing symptoms caused by the infection. The invention also relates to a fusion protein or a vaccine composition as aforementioned, for use in inducing enhanced pathogen antigen-specific T cell responses, or for use in killing a disease cell that presents an antigen via class I MHC molecules on the cell membrane of the disease cell, or for use in preventing, treating infection caused by a pathogen, and/or minimizing symptoms caused by the infection.

20 The pathogen may be at least one selected from the group consisting of Human Papillomavirus (HPV), Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), Human Immuno-deficient Virus (HIV-1), flu virus, Dangué virus, Hepatitis C virus (HCV), Hepatitis B virus (HBV) and Porcine Circovirus 2 (PCV2).

In one embodiment of the invention, the fusion protein as aforementioned is for use in enhancing an antigen-specific cytotoxic T cell response in a subject in need thereof. The fusion protein may also be for use in enhancing an antigen-specific CD4⁺ T cell response, or for use as an immunogenic enhancer for inducing an enhanced antigen-specific antibody titer response, in a subject in need thereof.

These and other aspects will become apparent from the following description of the preferred embodiment taken in conjunction with the following drawings, although variations and modifications therein may be affected without departing from the spirit and scope of the novel concepts of the disclosure.

The accompanying drawings illustrate one or more embodiments of the invention and, together with the written description, serve to explain the principles of the invention. Wherever possible, the same reference numbers are used throughout the drawings to refer to the same or like elements of an embodiment.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a vector map.

FIG. 2 is a photograph showing the result of SDS-PAGE analyses of fusion proteins.

FIG. 3 is a vector map.

FIG. 4 is a schematic drawing illustrating one embodiment of the invention.

FIG. 5A shows immunization schedules.

FIG. 5B is a photograph showing the result of SDS-PAGE analyses of fusion proteins.

FIGs. 5C-D are graphs showing tumor size curves and percentage of tumor-free mice in the animal groups vaccinated with various fusion proteins or placebo, respectively.

FIG. 6 shows a flow chart (left panel) for preparation of fusion proteins, and a photograph (right panel) showing the result of SDS-PAGE analyses of fusion proteins.

FIG. 7 is a schematic drawing illustrating the mechanisms of actions of T cell-sensitizing fusion proteins.

FIG. 8 shows sequence alignments of CD28 from various species.

FIG. 9A shows immunization schedules.

FIGs. 9B-C show tumor size curves and survival rate in the animal groups vaccinated with various fusion proteins or placebo, respectively.

FIG. 10 is a schematic drawing showing a RAP1-containing vector used for generating a plasmid containing a DNA insert from a pathogen.

FIG. 11 are schematic drawings illustrating constructions of fusion proteins containing antigens of various pathogens.

FIGs. 12A-F are photographs showing the results of SDS-PAGE analyses of various fusion proteins.

FIGs. 13A-B show animal groups, vaccines and dosage used for immunizing the animals, and immunization schedules.

FIGs. 14A-D are tables showing the results of *ex vivo* antigen-specific immune response analyses of CD3+/CD4+ splenocytes and CD3+/CD8+ splenocytes from the animal groups of FIG. 13A vaccinated with placebo or a fusion protein containing E7₁₆, E7₁₈, HCVcore, or HBx antigen.

FIGs. 15A-J show IFN γ + cell counts in *ex vivo* antigen-specific immune response analyses of CD3+/CD8+ splenocytes and CD3+/CD4+ splenocytes from the animal groups of FIG. 13A vaccinated with placebo or a fusion protein containing a PCV2 (15A-B) or PRRSV antigen (15C-J).

DETAILED DESCRIPTION OF THE INVENTION

The present invention is more particularly described in the following examples that are intended as illustrative only since numerous modifications and variations therein will be apparent to those skilled in the art. Various embodiments of the invention are now described in detail. Referring to the drawings, like numbers indicate like components throughout the views. As used in the description herein and throughout the claims that follow, the meaning of “a”, “an”, and “the” includes plural reference unless the context clearly dictates otherwise. Also, as used in the description herein and throughout the claims that follow, the meaning of “in” includes “in” and “on” unless the context clearly dictates otherwise. Moreover, titles or subtitles may be used in the specification for the convenience of a reader, which shall have no influence on the scope of the present invention. Additionally, some terms used in this specification are more specifically defined below.

DEFINITIONS

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

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. In the case of conflict, the present document, including definitions will control.

As used herein, “around”, “about” or “approximately” shall generally mean within 20 percent, preferably within 10 percent, and more preferably within 5 percent of a given value or range. Numerical quantities given herein are approximate, meaning that the term “around”, “about” or “approximately” can be inferred if not expressly stated.

The term “an antigen-presenting cell (APC) or accessory cell” refers to a cell that displays foreign antigens complexed with major histocompatibility complexes (MHC's) on their surfaces. T-cells may recognize these complexes using their T-cell receptors (TCRs). These cells process antigens and present them to T-cells. Main types of professional antigen-presenting cell: dendritic cells (DCs), macrophages, monocytes, and certain B-cells.

The term “an antigen-presenting cell (APC)-binding domain” refers to a domain that can bind to an antigen-presenting cell (APC). The APC-binding domain may be a polypeptide comprising an amino acid sequence that is at least 90% identical to the sequence selected from the group consisting of SEQ ID NOs: 5, 6, 7, 8, and 9. An APC-binding domain is a ligand that recognizes and binds to a receptor on APC.

Cluster of differentiation 91 (CD91) is a protein that forms a receptor in the membrane of cells and is involved in receptor-mediated endocytosis.

The term “a protein transduction domain” refers to a polypeptide or a fusion polypeptide having a function to sensitize T-cells and thus enhance antigen-specific T cell responses, and/or to guide or direct an antigen toward (i.e., to target to) class I major histocompatibility complex (MHC-I) pathway (i.e., a cytotoxic T cell pathway) of antigen presentation.

The term “to sensitize T cells” generally means that CD8⁺ and CD4⁺ T cells are sensitized and as a result, CD8⁺ (CTL) and CD4⁺ T cell responses to an antigen challenge are enhanced. An antigen-specific cell mediated immune response is measured by quantifying the production of antigen-specific induced γ -interferon in response to an antigen. For example, without a sensitization signal (i.e., without the protein transduction domain), an antigen alone may induce weak or no cell mediated immune response at all, i.e., weak or no production of antigen-specific γ -interferon from CD8⁺ and CD4⁺ T cells, while in the presence of a sensitization signal (the protein transduction domain), the antigen may induce an enhanced cell mediated immune response. Thus, the function of a sensitization signal (the

protein transduction domain) is to sensitize CD4⁺ and CD8⁺ T cells in a host so that when the host is later challenged by an antigen, the antigen can induce an enhanced antigen-specific cell mediated immune response due to prior CD4⁺ and CD8⁺ T cell sensitization.

A protein transduction domain may be a peptide and/or polypeptide selected from the group consisting of:

(i) a fusion polypeptide, comprising a T cell sensitizing signal-transducing peptide, a linker, and a translocation peptide, wherein:

(1) the T cell sensitizing signal-transducing peptide is located at the N-terminus of the fusion polypeptide;

(2) the linker comprises SEQ ID NO: 15, linking the T cell sensitizing signal-transducing peptide and the translocation peptide; and

(3) the translocation peptide has 34-112 amino acid residues in length and comprises the amino acid sequence that is at least 90% identical to SEQ ID NO: 3, 20 or 4;

(ii) a T cell-sensitizing signal-transducing peptide; and

(iii) a translocation peptide of 34-112 amino acid residues in length, comprising the amino acid sequence that is at least 90% identical to SEQ ID NO: 3, 20 or 4.

A protein transduction domain may be “a fusion polypeptide”, in which the fusion polypeptide comprises a T cell sensitizing signal-transducing peptide, a linker, and a translocation peptide. For example, the fusion polypeptide may be the polypeptide “CD28convPE_t”.

The term “CD28conv” refers to a CD28 conserved region, which is a “T cell sensitizing signal-transducing peptide”. It's an epitope for inducing CD28 agonist antibody.

The term “PE_t” refers to a translocation peptide with 34-112 amino acid residues in length.

A linker is present between the “CD28conv” and the “PE_t”. The orientation or arrangement of the fusion polypeptide “CD28convPE_t” is important in that “CD28conv” (or the T cell sensitizing signal-transducing peptide) must be at the upstream to the PE_t (or the translocation peptide), i.e., PE_t must be at the C-terminus of the “CD28conv” to obtain enhanced T-cell responses. The “CD28convPE_t” can raise much higher IgG titer (called CD28-specific agonist antibody) specific to CD28conv than the reversed orientation fusion peptide PE_tCD28conv. The CD28-specific agonist antibody can sensitize both CD4⁺ and CD8⁺ T cells. The correct orientation fusion polypeptide CD28convPE_t contains a linker (RXRXKR) between CD28conv and PE_t domains. The linker contains an antigen presenting cell (APC)-specific protease (cathepsin L) cutting site Lys-Arg (KR). Therefore, the fusion protein RAPI-

CD28convPE_t-Antigen-K3 can be digested into the two fragments: RAP1-CD28conv and PE_t-Antigen-K3. The RAP1-CD28conv fragment can be further digested in the lysosome and the epitope of CD28conv is then presented to the APC cell surface via MHC II pathway, which in turn elicits a humoral immune response producing CD28 agonist antibody. Thus, CD28 agonist antibody is produced by B cells. This CD28 agonist antibody can bind to CD28 on the T cell surface and pre-activate the T cells (CD4+ and CD8+ T cells).

A "T cell-sensitizing signal-transducing peptide" has 28-53 amino acid residues in length and comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 31, in which Xaa⁸ is I or L; Xaa¹⁰ is V, F or A, Xaa¹¹ is M or L, Xaa¹⁷ is L or I.

The T cell-sensitizing signal-transducing peptide comprises the critical region K¹(I/L)²E³(V/F/A)⁴(M/L)⁵Y⁶P⁷P⁸P⁹Y¹⁰ (SEQ ID NO: 32), wherein (X)² is I or L; (X)⁴ is V, F or A, (X)⁵ is M or L.

A T cell sensitizing signal-transducing peptide (TDIYFCKIEFMYPYLDNEKSNGTHH; SEQ ID NO: 31, wherein X⁸ is I, X¹⁰ is F, X¹¹ is M) specific for mice was used in the following examples.

FIG. 7 is a schematic drawing illustrating the mechanisms of actions of T cell-sensitizing fusion proteins using the fusion protein RAP1-CD28convPE_t-E7-K3 as an example. RAP1-CD28convPE_t-E7-K3 comprises from N-terminus to the C-terminus: (1) the domain III of the full length RAP1 at the N-terminus, (2) CD28conv, (3) a linker, (4) a modified translocation peptide from Pseudomonas Exotoxin A, (5) a full length HPV type 16 E7 protein, and (6) the triple KDEL as an ER retention signal at the C-terminus. The HPV16 E7 protein is processed into epitopes within the cells of a subject immunized with the fusion protein. The RAP1-CD28convPE_t-E7-K3 can elicit a better cytotoxic T-cell (CTL) response than the traditional vaccines that contain antigen only. RAP1-CD28convPE_t-E7-K3 protein was designed to improve APC (such as dendritic cell) uptake efficiency and enhance HPV16 E7 antigen processing toward a proteasome pathway, then presented via MHC I complexes. The mechanism of action of HPV16 E7 protein-specific CTL immune response elicited by the vaccine RAP1-CD28convPE_t-E7-K3 is illustrated in FIG. 7: (a) the vaccine binds to APC (such as dendritic cell) surface receptor (CD91) and internalized via endocytosis; (b1) RAP1-CD28convPE_t-E7-K3 undergoes proteolytic hydrolysis by cathepsin L protease digestion at the site before the translocation peptide PE_t; (b2) or recycles to the E.R. and undergoes proteolytic hydrolysis by furin protease at the site before the translocation peptide PE_t; (b3) At the meantime, RAP1-CD28conv was digested by lysosomal protease and the epitopes of CD28conv are then presented to the cell surface via MHC II and elicits

CD28 agonist antibody production, which can pre-active T cells; (c) The most important step is transmembrane translocation of the PE_t-E7-K3 into the cytoplasmic compartment from the lysosome by translocation peptide (PE_t); (d) the PE_t-E7-K3 undergoes digestion via proteasome pathway, then the epitopes of E7 are presented by MHC I complex and elicit E7-specific cell mediated immune response.

FIG. 8 shows a sequence alignment of CD28 conserved regions from various species and the consensus sequence. The underlined sequence (KIEVMYPPPY; SEQ ID NO: 32, where X² is I, X⁴ is V, X⁵ is M) within the consensus sequence is a critical region for CD28 agonist antibody recognition and binding. This critical region sequence can be represented by K¹(I/L)²E³(V/F/A)⁴(M/L)⁵Y⁶P⁷P⁸P⁹Y¹⁰, in which only the fourth amino acid residue therein is species-specific and should be V in human, rat, porcine, bovine, sheep, dog and horse; F in mouse, and V in turkey. The critical region sequence may be represented as K¹(X)²E³(X)⁴(X)⁵Y⁶P⁷P⁸P⁹Y¹⁰ (SEQ ID NO: 32), wherein (X)² is I or L; (X)⁴ is V, F or A, (X)⁵ is M or L.

PE_t may comprise the amino acid sequence that is at least 90% identical to SEQ ID NO: 3 or 20. For example, the amino acid sequence of PE_t may be a.a. 280 - a.a. 313 (SEQ ID NO: 3), a.a. 268 - a.a. 313 (SEQ ID NO: 20), a.a. 253 - a.a. 313, or a.a. 253 - a.a. 364 (SEQ ID NO: 4) of PE. That is, the amino acid sequence of PE_t may contain any region of the PE domain II (a.a. 253 to a.a. 364; SEQ ID NO: 4) as long as it comprises a.a. 280-a.a. 313 (SEQ ID NO: 3) essential fragment.

An antigen may be a pathogenic protein, polypeptide or peptide that is responsible for a disease caused by the pathogen, or is capable of inducing an immunological response in a host infected by the pathogen, or tumor-associated antigen (TAA) which is a polypeptide specifically expressed in tumor cells. The antigen may be selected from a pathogen or cancer cells including, but not limited to, Human Papillomavirus (HPV), PRRSV, HIV-1, flu virus, Danguue virus, Hepatitis C virus (HCV), Hepatitis B virus (HBV), Porcine Circovirus 2 (PCV2), non-small cell lung cancer, breast carcinoma, melanoma, lymphomas, colon carcinoma, hepatocellular carcinoma and any combination thereof. For example, HPV E7 protein (E7), HCV core protein (HCV core), HBV X protein (HBx) were selected as antigens for vaccine development. The antigen may be a fusion antigen from a fusion of two or more antigens selected from one or more pathogenic proteins. For example, a fusion antigen of PRRSV ORF6 and ORF5 fragments, or a fusion of antigenic proteins from PRRSV and PCV2 pathogens.

The function of an endoplasmic reticulum retention sequence is to assist translocation of an antigen from an endocytotic compartment into ER and retains it in the lumen. It comprises the sequence Lys Asp Glu Leu (KDEL) or RDEL. An ER sequence may comprise, or consists essentially of, or consist of, the

sequence of KKDLRDELKDEL (SEQ ID NO: 16), KKDELRDELKDEL (SEQ ID NO: 17),
KKDELRVELKDEL (SEQ ID NO: 18).

Receptor-associated protein (RAP1) with a molecular weight of 39 kDa is an ER resident protein and molecular chaperone for LDL receptor-related protein. It has a high binding affinity to CD91 (Kd~ 3 nM) and is composed by three functional-similar domains.

The invention relates to the discovery of induction and enhancement of T cell mediated immune responses by fusion proteins according to the invention. Using RAP1-CD28convPE₄-E7-K3 an example, the strategy of RAP1-CD28convPE₄-E7-K3 vaccine is focused primarily on stimulating the production and activation of T cells that can recognize HPV16 infected cells expressing the target antigen E7. By delivering antigens to dendritic cells, it can generate antigen-specific CD8⁺ T cells and CD4⁺ T cells. Type 1-helper CD4⁺ T cells particularly are able to efficiently stimulate and augment the immune response of cytotoxic CD8⁺ T cells. Together, these two arms of the adaptive immune system have the specificity and potency to kill HPV16-infected cells or HPV16-associated tumor cells at multiple sites in the body without inflicting significant damage on normal tissues.

The term "subject" refers to a human or a non-human animal.

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

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

Abbreviations: CD 28, Cluster of Differentiation 28.

EXAMPLES

Without intent to limit the scope of the invention, exemplary instruments, apparatus, methods and their related results according to the embodiments of the present invention are given below. Note that titles or subtitles may be used in the examples for convenience of a reader, which in no way should limit the scope of the invention. Moreover, certain theories are proposed and disclosed herein; however, in no way they, whether they are right or wrong, should limit the scope of the invention so long as the

invention is practiced according to the invention without regard for any particular theory or scheme of action.

EXAMPLE 1

Constructions of Expression Vectors

FIG. 1 shows the expression vector for the fusion protein RAP1- PE₂₆₈₋₃₁₃-E7-K3, which comprises a RAP1 domain 3 (SEQ ID NO: 5), a translocation minimum essential peptide (PE₂₆₈₋₃₁₃; SEQ ID NO: 20), the antigen E7, and an endoplasmic reticulum retention sequence (K3, or a KDEL signal; SEQ ID NO: 16, 17, 18, or 19). The translocation minimum essential peptide (PE₂₆₈₋₃₁₃; SEQ ID NO: 20) was obtained from PE (SEQ ID NO: 10) polypeptide sequence region from a.a. 268 to a.a. 313.

This expression vector was constructed by using the plasmid RAP1-K3 shown in FIG. 10. The plasmid RAP1-K3 (FIG. 10), which contains a fusion gene of RAP-1 domain 3 and K3, was generated as follows: A DNA fragment encoding ^{NdeI}RAP1-(EcoRI, XhoI)-K3^{XhoI} was synthesized by a PCR method and ligated into the plasmid pUC18 backbone with kanamycin resistance gene to obtain the plasmid RAP1-K3 (FIG. 10). To generate the expression vector RAP1-PE₂₆₈₋₃₁₃-E7-K3 (FIG. 1), a DNA fragment encoding PE₂₆₈₋₃₁₃-E7 was inserted into the plasmid RAP1-K3 (FIG. 10). Using similar methods, DNA fragments encoding various fusion peptides were generated by PCR (FIG. 11) and inserted, respectively, into the plasmid RAP1-K3 (FIG. 10) to generate expression vectors for various fusion proteins (FIGs. 12A-F). FIG. 3 illustrates the expression vector for the fusion protein RAP1-CD28convPE₁-E7-K3 (FIG. 3).

The fragment CD28convPE₁ contains cathepsin L and furin protease cutting sites. FIG. 4 shows an amino acid sequence map of the fusion protein RAP1-CD28convPE₁-E7-K3 to illustrate the importance of the fragment CD28convPE₁. The two arrows indicate cathepsin L and furin protease cutting sites, respectively. These two cutting sites not only serve as linkers to ligate CD28conv and PE₁-E7 but also allow cutting of the fusion protein to release the fragment PE₁-E7-K3 to the cytoplasm from the lysosome. Any other antigens of interest from various pathogens may replace E7 to fuse with CD28convPE₁ and the fusion product can then be inserted into the plasmid of FIG. 10 to make a variety of expression vectors for fusion proteins illustrated in FIG. 11.

The sequence of PE₂₆₈₋₃₁₃ is: pletftrhrqprgweqleqcgypvqrlylaarlswnqvddvir (SEQ ID NO: 20). The whole sequence of CD28convPE₁ is as follows:

tdiyfckiefmypppyldneksngtihrarykrgweqleqcgypvqrlylaarlswnqvddvirgs (SEQ ID NO: 30), the sequence underlined represents a linker sequence containing Cathepsin L and furin protease cutting sites.

EXAMPLE 2

Protein expression

E. coli BL21 cells harboring a protein expression vector were cultured in Luria Bertani broth containing 25 µg/ml kanamycin at 37°C. When the culture reached an early log phase, (A600=0.1 to 0.4), isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added at a final concentration of 0.5 to 2 mM for induction. Cells were harvested after 4 hours IPTG induction and disrupted by sonication. The overexpressed protein-containing inclusion bodies were isolated and solubilized in 8M urea/TN buffer (8M urea, 50mM Tris, 50mM NaCl, pH 8.0).

The refolding of the fusion protein RAP1-PE₂₆₈₋₃₁₃-E7-K3 was performed by dialysis against 50X volume of TNZ buffer (50mM Tris, 50mM NaCl and 0.01mM ZnCl₂, pH 8.0) at 4°C overnight. The refolded proteins were subject to SDS-PAGE analyses under reduced (with dithiothreitol; +DTT) and non-reduced (without dithiothreitol; -DTT) conditions (FIG. 2). The results indicated that most of the refolded proteins were monomers under a non-reduced condition, indicating that the RAP1 fusion protein refolded easily and were not aggregated (FIG. 2).

FIG. 6A is a flow chart illustrating that the fusion proteins RAP1-CD28PE_T-E7-K3 (with mouse CD28conv or human CD28conv) were expressed and extracted from the inclusion bodies of *E. coli* cells). SDS-PAGE analyses indicated that the fusion proteins refolded well (FIG. 6B).

FIG. 11 illustrates a list of fusion proteins that were expressed using similar method described above:

(1) RAP1-PE₂₆₈₋₃₁₃-E7-K3; (2) RAP1-CD28convPE_T-E7-K3; (3) RAP1-CD28PE_T-E7₁₈-K3; (4) RAP1-HCVcore-K3; (5) RAP1-CD28conv-HCVcore-K3; (6) RAP1-CD28convPE_T-HCVcore-K3; (7) RAP1-HBx; (8) RAP1-HBx-K3; (9) RAP1-CD28conv-HBx; (10) RAP1-CD28conv-HBx-K3; (11) RAP1-CD28convPE_T-HBx; (12) RAP1-CD28convPE_T-HBx-K3; (13) RAP1-PCV2_{ORF2}-K3; (14) RAP1-PE₂₆₈₋₃₁₃-PCV2_{ORF2}-K3; (15) RAP1-CD28convPE_T-PCV2_{ORF2}-K3; (16) RAP1-PE₂₆₈₋₃₁₃-DGD-K3; (17) RAP1-PE₂₆₈₋₃₁₃-M12-K3; (18) RAP1-PE₂₆₈₋₃₁₃-PQAB-K3; (19) RAP1-PE₂₆₈₋₃₁₃-RSAB-K3; (20) RAP1-CD28convPE_T-DGD-K3; (21) RAP1-CD28convPE_T-M12-K3; (22) RAP1-CD28convPE_T-PQAB-K3; (23) RAP1-CD28convPE_T-RSAB-K3. These fusion proteins were refolded using the same method described above. The results of SDS-PAGE analyses indicated these fusion proteins all refolded well and were thus used for preparing vaccines (FIGs. 12A-F).

EXAMPLE 3

RAP1-PE₂₆₈₋₃₁₃-E7-K3 inhibits growth of Tumors induced by human papilloma virus (HPV) type 16 E7 protein

The fusion proteins PE₄₀₇-E7-K3 and RAP1-PE₂₆₈₋₃₁₃-E7-K3 were expressed as described above and protein refolding examined by SDS-PAGE (FIG. 5B). Mice were challenged with 2×10^3 TC-01 cells (a mouse lung epithelia cell line harboring HPV type 16 E7 gene) via s.c. injection to induce HPV-16 type carcinoma. Twelve days after the TC-01 cell challenge, mice were vaccinated via s.c. with placebo (PBS + aluminum phosphate), PE₄₀₇-E7-K3 (200 µg/dose) or RAP1-PE₂₆₈₋₃₁₃-E7-K3 (200 µg/dose) with AS04C (GlaxoSmithKline) as an adjuvant once per week for 3 weeks (FIG. 5A). AS04C, which is a cytotoxic T lymphocyte-enhancing adjuvant, comprises MPL (monophosphoryl lipid A, an immune potentiator) and aluminum phosphate (a protein absorbent for antigen delivery). The term "K3" stands for the amino acid sequence KDELKDELKDEL (SEQ ID NO: 19). The size of tumors and the number of tumor-free animals in each group were recorded (FIGs. 5C-D). The tumor growth was significantly suppressed by both vaccines PE₄₀₇-E7-K3 and RAP1-PE₂₆₈₋₃₁₃-E7-K3 with AS04C as an adjuvant. However, the mouse group vaccinated with RAP1-PE₂₆₈₋₃₁₃-E7-K3 had a higher rate of tumor-free mice. This indicated that the vaccine RAP1-PE₂₆₈₋₃₁₃-E7-K3 was as effective as or better than PE₄₀₇-E7-K3 in suppressing tumor growth, however was better in increasing the percentage of tumor-free animals.

EXAMPLE 4

RAP1-CD28convPE_r-E7-K3 inhibits growth of Tumors induced by human papilloma virus (HPV) type 16 E7 protein and increases survival rate

The effects of the fusion proteins PE₄₀₇-E7-K3 and RAP1-CD28convPE_r-E7-K3 with or without an immune potentiator on tumor size and survival rate were examined. Mice were challenged with a higher dose of TC-01 cells (3×10^4) via s.c. injection. Seven days after the challenge, mice were vaccinated via s.c. with placebo, PE₄₀₇-E7-K3 (100 µg/dose) or RAP1-CD28convPE_r-E7-K3 (100 µg/dose) with the immune potentiator GPI-0100 or the protein absorbent aluminum phosphate once per week for 3 weeks (FIG. 9A). GPI-0100 is a Th1/CTL stimulating adjuvant (immune potentiator). The size of the tumors and the survival rate in each group were recorded (FIGs. 9B-C). When combined with the adjuvant GPI-0100, both PE₄₀₇-E7-K3 and RAP1-CD28convPE_r-E7-K3 suppressed the tumor growth. Unexpectedly, it was discovered that the effect of RAP1-CD28convPE_r-E7-K3 in inhibiting tumor growth was not dependent on the adjuvant. When combined with the absorbent aluminum phosphate rather than with the adjuvant GPI-0100, RAP1-CD28convPE_r-E7-K3 could still significantly suppress the tumor growth with the same potency as that when combined with the immune potentiator GPI-0100 (FIG. 9B, unfilled triangle v. filled reverse triangle).

In contrast, the potency of PE₄₀₇-E7-K3 in suppressing the tumor growth depended on the adjuvant. When combined with the absorbent aluminum phosphate, PE₄₀₇-E7-K3 became less potent than that when combined with the immune potentiator GPI-0100 (FIG. 9B solid square v. unfilled circle).

On the other hand, mice administrated with RAP1-CD28convPE_t-E7-K3 in combination with the immune potentiator GPI-0100 or the absorbent aluminum phosphate had a better survival rate than the groups vaccinated with PE₄₀₇-E7-K3 in combination with GPI-0100 or aluminum phosphate (FIG. 9C). This indicated that RAP1-CD28convPE_t-E7-K3 could elicit Th1/CTL immune responses even without the immune potentiator GPI-0100. The results also indicated that the fusion protein RAP1-CD28convPE_t-E7-K3 was superior to PE₄₀₇-E7-K3 as a vaccine for increasing the survival rate of the animals.

EXAMPLE 5

Immunogenicity assays

The immunogenicities of various vaccines were tested. Briefly, mice were divided into the following groups: HPV16 E7, HPV 18 E7, HCV core, HBV HBx, PCV2 ORF2 and PRRSV (FIG. 13A). Each group was further divided into subgroups, and each subgroup was injected with either a placebo or a vaccine designed to target toward a certain antigen, or certain antigens, of a pathogen via s.c. once per week for 3 weeks (FIG. 13B). Except the vaccines targeted to PRRSV, each vaccine was composed of a single fusion protein and the absorbent aluminum phosphate, in which the single fusion protein contained at least an antigen of a pathogen. The antigen was either a full-length protein from a pathogen, or a non-full-length protein that contained at least one epitope of an antigen of a pathogen, or was a fusion peptide of two or more antigens, in which each of the antigens was selected from different proteins of a pathogen.

The immunization schedule, vaccines and dose are illustrated in FIGs. 13A-B. Briefly, mice were vaccinated once per week for 3 weeks with vaccines listed in FIG. 13A. All mice were sacrificed 7 days after the last immunization, and the spleens were harvested. Splenocytes were isolated and cultured in 6-well plate (2×10^7 cells/2ml/well) with 10 µg/ml of respective recombinant antigens of pathogens to stimulate the splenocytes in the presence of 1 µg/ml GolgiPlug (BD Pharmingen, San Diego, CA) at 37°C for 16 hr.

The stimulated splenocytes were washed with FACScan buffer and the cell surface markers CD8a, CD4, and CD3 were stained with phycoerythrin-conjugated monoclonal rat anti-mouse CD8a, AF700-conjugated monoclonal rat anti-mouse CD4 and AF647-conjugated monoclonal rat anti-mouse CD3

antibodies. The cells were then permeabilized and fixed by Cytofix/Cytoperm kit according to the manufacturer's instructions (BD Pharmingen). Intracellular IFN- γ was stained with AF488-conjugated rat anti-mouse IFN- γ to measure the immune response and cytokine levels. Flow cytometry analyses were performed using Gallios flow cytometry with Kaluza analysis software (Beckman Coulter).

5 The following PRRSV vaccines were tested for immunogenicities: PE₄₀₇-PRRSV-K3, RAP1-PE₂₆₈₋₃₁₃-PRRSV-K3 or RAP1-CD28convPE₁-PRRSV-K3 vaccine. Each vaccine contained a mixture of four different fusion proteins, and each fusion protein contained a different antigen that is selected from the group consisting of DGD, M12, PQAB and RSAB (FIG. 13A). Vaccination of mice and stimulations of splenocytes were performed using similar method as described above. Briefly, all mice were sacrificed 7
10 days after the last immunization, and spleens were harvested. The splenocytes were isolated and cultured in 6-well plate (2×10^7 cells/2ml/well) with 10 μ g/ml of the recombinant DGD, M12, PQAB or RSAB antigens, separately, to stimulate the splenocytes in the presence of 1 μ g/ml GolgiPlug (BD Pharmingen, San Diego, CA) at 37°C for 16 hr.

The amino acid sequence of "DGD" (SEQ ID NO: 26) is as follows:

15 **RHHFTP****SERQLCLSSIQTAFNQAGATC****ILSDSGRISYTFEFS****LPHTHTVRLIRVTAPPS**
ALDOVIRNALASPGSGGDLGEAIREOPEQARLALTAAAESERFVROGTGNDEAGANADVVS
LTC**PVAAGECAGPADSGDALLERNYPTGA****EFLGDGGDVR****RHHFTP****SERQLCLSSIQTAFNQGA**
GTCILSDSGRISYTFEFS**LPHTHTVRLIRVTAPPSA**. DGD represents a fusion antigen of PRRSV
ORF7 a.a. 64 - a.a. 123 (boldface), linker (underlined) and ORF7 a. a. 64- a.a. 123 (boldface).

20 The term "M12" represents a antigen of PRRSV ORF1b a.a. 1046 - a.a. 1210. Its amino acid sequence (SEQ ID NO: 27) is as follows:

NNKECTVAQALGNGDKFRATDKRVVDSLRAICADLEGSSSPKVAHNLGIFYFSPDLTQFAKL
PIELAPHWPVVSTQNNEKWPDLVASLRPLDKYSRACIGAGYMGPSVFLGTPGVVSYYLTKF
VKGEAQVLPETVFSTGRIEVDCREYLDDREREVAASLPH.

25 The amino acid sequence of "PQAB" (SEQ ID NO: 28) is as follows:

GSSLDDFCYDSTAPQKVLLAFSITYASNDSSSHLQLIYNLTICELNGTDWLANKFDWA. PQAB
represents a fusion antigen of PRRSV American strain ORF6 a.a. 2- a.a. 26 and ORF5 a.a. 31- a.a. 63
(underlined).

The amino acid sequence of RSAB is

30 MGSLDDFCNDSTAAQKLVLAFSITYTPIFVAGGSSSTYQYIYNLTICELNGTDWLSNHFDWA
(SEQ ID NO: 29). The term "RSAB" represents a fusion antigen of PRRSV European strain ORF6 a.a.

2-28 and ORF5 a.a. 31-64 (underlined).

EXAMPLE 6

The fragment of RAP1 domain 3 of the fusion protein RAP1-CD28convPE_E-E7-K3 is replaced by A2M minimum (SEQ ID NO: 6), HIV-Tat minimum (SEQ ID NO: 7) or HSPs minimum (SEQ ID NO: 8) to generate the fusion proteins A2M- CD28convPE_E-E7-K3, Tat-CD28convPE_E-E7-K3 and HSP-CD28convPE_E-E7-K3 vaccines, respectively. The TC-1 tumor suppression activity and cell mediated immune responses enhanced by these vaccines are examined using similar methods as described above. Table 1 shows SEQ ID NOs. of the components of various fusion proteins. Table 2 shows the fusion proteins tested for the effects on T cell-mediated immune responses in animals and the sequences of antigens.

Table 1

Component	SEQ ID NO:	Length (residues)
hCD28 Core TDIYFCKIEVMYPPPYLDNEKSNGTIIH	1	28
hCD28 Maximum NCDGKLGNESVTFYLNLYVNQTDIYFCKIEVMYPPPYLDNE KSNGTIIHVKG	2	53
PE _E Core (PE translocation domain core; a.a. 280- a.a. 313 of PE)	3	34
PE _E Maximum (translocation domain maxi, a.a. 253 - a.a. 364 of PE)	4	112
RAP1 Minimum (domain III of RAP1)	5	104
A2M Minimum	6	153
HIV-Tat Minimum	7	24
HSPs Minimum, Heat shock 70 kDa protein (HSPs; Homo sapiens)	8	641
Minimum Pseudomonas exotoxin A (PE) binding domain Ia (an APC-binding domain, a.a. 1- a.a. 252 of PE)	9	252
Linker RXXRKR,), in which "X" is any amino acid residue.	15	6
Full length PE (Exotoxin A mature form, <i>Pseudomonas aeruginosa</i>)	10	613
Full length RAP1 (<i>Homo sapiens</i> low density lipoprotein receptor-related protein associated protein 1, LRPAP1); Domain 1: a.a. 1- a.a.	11	323

112; domain 2: a.a. 113 - a.a. 218; domain 3: a.a. 219 - a.a. 323.		
Full length A2M (<i>Homo sapiens</i> alpha-2-macroglobulin receptor-associated protein precursor)	12	357
HIV-Tat (Human immunodeficiency virus 1)	13	101
KDEL	14	4
KKDLRDELKDEL	16	12
KKDELRLDELKDEL	17	13
KKDELRLVELKDEL	18	13
KDELKDELKDEL	19	12
PE ₂₆₈₋₃₁₃ PLETFTRHRQPRGWEQLEQCGYPVQRLVALYLAARLSWNQV DQVIR	20	46
CD28convPE _i T ¹ D ² I ³ Y ⁴ F ⁵ C ⁶ K ⁷ X ⁸ E ⁹ X ¹⁰ X ¹¹ Y ¹² P ¹³ P ¹⁴ P ¹⁵ Y ¹⁶ X ¹⁷ D ¹⁸ N ¹⁹ E ²⁰ K ²¹ S ²² N ²³ G ²⁴ T ²⁵ I ²⁶ I ²⁷ H ²⁸ R ²⁹ X ³⁰ R ³¹ X ³² K ³³ R ³⁴ G ³⁵ W ³⁶ E ³⁷ Q ³⁸ L ³⁹ E ⁴⁰ Q ⁴¹ C ⁴² G ⁴³ Y ⁴⁴ P ⁴⁵ V ⁴⁶ Q ⁴⁷ R ⁴⁸ L ⁴⁹ V ⁵⁰ A ⁵¹ L ⁵² Y ⁵³ L ⁵⁴ A ⁵⁵ A ⁵⁶ R ⁵⁷ L ⁵⁸ S ⁵⁹ W ⁶⁰ N ⁶¹ Q ⁶² V ⁶³ D ⁶⁴ Q ⁶⁵ V ⁶⁶ I ⁶⁷ R ⁶⁸ , wherein (X) ⁸ is I or L; (X) ¹⁰ is V, F or A, (X) ¹¹ is M or L, X ¹⁷ is L or I, (X) ^{30, 32} is any amino acid residue.	30	68
CD28 consensus sequence T ¹ D ² I ³ Y ⁴ F ⁵ C ⁶ K ⁷ (X) ⁸ E ⁹ (X) ¹⁰ (X) ¹¹ Y ¹² P ¹³ P ¹⁴ P ¹⁵ Y ¹⁶ X ¹⁷ D ¹⁸ N ¹⁹ E ²⁰ K ²¹ S ²² N ²³ G ²⁴ T ²⁵ I ²⁶ I ²⁷ H ²⁸ , wherein (X) ⁸ is I or L; (X) ¹⁰ is V, F or A, (X) ¹¹ is M or L, X ¹⁷ is L or I.	31	28
CD28 critical region K ¹ (X) ² E ³ (X) ⁴ (X) ⁵ Y ⁶ P ⁷ P ⁸ P ⁹ Y ¹⁰ , wherein (X) ² is I or L; (X) ⁴ is V, F or A, (X) ⁵ is M or L.	32	10

Table 2

Fusion protein name	Antigen Name	Antigen SEQ ID NO:
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RAP1-CD28convPE _t -E7-K3	HPV16 E7 (full length)	21
RAP1-CD28convPE _t -E7 ₁₈ -K3	HPV18 E7 (full length)	22
RAP1-CD28convPE _t -HCVc-K3	HCV core protein (full length)	23
RAP1-CD28convPE _t -HBX-K3	HBV X protein (full length)	24
RAP1-CD28convPE _t -PCV2-K3	PCV2 ORF2 (a fragment of ORF2)	25
RAP1-CD28convPE _t -DGD-K3	PRRSV nucleocapsid (a fusion antigen: ORF7 a.a. 64 - a.a. 123, linker and ORF7 a.a. 64- a.a. 123)	26
RAP1-CD28convPE _t -M12-K3	PRRSV RNA-dependent RNA polymerase (ORF1b a.a.1046-a.a. 1210)	27
RAP1-CD28convPE _t -PQAB-K3	PRRSV American strain: a fusion antigen of ORF6 (a.a. 2-a.a. 26) and ORF5 (a.a. 31- a.a. 63)	28
RAP1-CD28convPE _t -RSAB-K3	PRRSV European strain: a fusion antigen of ORF6 (a.a. 2-a.a. 28) and ORF5 (a.a. 31-a.a. 64)	29

In the immunogenicity assays, antigen-specific cell-mediated immune responses induced by various vaccines were evaluated by measuring the numbers of CD3+/CD4+/IFN γ + and CD3+/CD8+/IFN γ + T cells in the splenocytes. The results indicated that the vaccine RAP1-CD28convPE_t-antigen-K3 can induce strong T cell responses. FIG. 14 B shows the CD3+/CD4+/IFN γ + T cell number and the CD3+/CD8+/IFN γ + T cell number elicited by CD28convPE_t-E7₁₈-K3 were about 50 times and greater than 9 times of RAP1-K3, respectively.

The vaccine RAP1-CD28convPE_t-antigen-K3 is superior to PE₄₀₇-antigen-K3 in eliciting T cell-mediated immunogenicity. For example, FIG. 14A illustrates the CD3+/CD4+/IFN γ + T cell number and the CD3+/CD8+/IFN γ + T cell number elicited by CD28convPE_t-E7₁₆-K3 were about 5 times and 7 times of PE₄₀₇-E7₁₆-K3, respectively. This indicates that the vaccine RAP1-CD28convPE_t-E7-K3 had a better cell-mediated immunogenicity than PE₄₀₇-E7-K3.

A fusion protein comprising RAP1 domain III, the sensitizing signal CD28conv alone without the translocation peptide PE_t, antigen and an ER retention signal is sufficient in eliciting a strong antigen-

specific T cell mediated immune responses when the antigen chosen comprises ten or greater than 10 epitopes. FIG. 14C illustrates the vaccine RAP1-CD28conv-HCVcore-K3 elicited T cell responses with the numbers of CD3+/CD4+/IFN γ + and of CD3+/CD8+/IFN γ + T cells being 20 times and 7.6 times of the placebo group, respectively. The antigen HCVcore contains 11 well-known MHC I epitopes.

5 It was unexpected that the ER retention signal is not essential for the fusion protein of the invention to elicit a strong cell-mediated immunogenicity. In other words, without the ER retention sequence, the fusion protein of the invention can still elicit strong T-cell responses. FIG. 14D illustrates that the numbers of CD3+/CD4+/IFN γ + and CD3+/CD8+/IFN γ + T cells elicited by RAP1-CD28convPE₁-HBx (without the ER retentions signal K3) were 7 times and 74 times of the placebo group.

10 In contrast, U.S. Patent Numbers 7378100B2 and 7335361 show that the ER retention signal K3 is indispensable for PE-related fusion proteins (PE₄₀₇-antigen-K3) to elicit T cell responses.

It was also discovered that a fusion protein comprising RAP1 domain III, the translocation peptide PE₂₁₈₋₃₁₃ (without the sensitizing signal CD28conv), antigen and an ER retention signal is superior to a PE-related fusion protein without containing the RAP1 domain III. FIG 15C-J illustrate the vaccine
15 RAP1-PE₂₆₈₋₃₁₃-PRRSV-K3 elicited greater CD3+/CD4+/IFN γ + and CD3+/CD8+/IFN γ + T cell counts than the vaccine PE₄₀₇-PRRSV-K3.

The foregoing description of the exemplary embodiments of the invention has been presented only for the purposes of illustration and description and is not intended to be exhaustive or to limit the invention to the precise forms disclosed. Many modifications and variations are possible in light of the
20 above teaching. The embodiments and examples were chosen and described in order to explain the principles of the invention and their practical application so as to enable others skilled in the art to utilize the invention and various embodiments and with various modifications as are suited to the particular use contemplated. Alternative embodiments will become apparent to those skilled in the art to which the present invention pertains without departing from its spirit and scope. Accordingly, the scope of the
25 present invention is defined by the appended claims rather than the foregoing description and the exemplary embodiments described therein.

CLAIMS

What is claimed is:

- 5 1. A fusion protein comprising:
 - (a) an antigen-presenting cell (APC)-binding domain or a CD91 receptor-binding domain, located at the N-terminus of the fusion protein;
 - (b) a protein transduction domain, located at the C-terminus of the APC-binding domain or the CD91 receptor-binding domain, the protein transduction domain being selected from the group
 10 consisting of:
 - (i) a fusion polypeptide, comprising a T cell sensitizing signal-transducing peptide, a linker, and a translocation peptide, wherein:
 - (1) the T cell sensitizing signal-transducing peptide is located at the N-terminus of the fusion polypeptide;
 - 15 (2) the linker comprises SEQ ID NO: 15, linking the T cell sensitizing signal-transducing peptide and the translocation peptide; and
 - (3) the translocation peptide has 34-112 amino acid residues in length and comprises the amino acid sequence that is at least 90% identical to SEQ ID NO: 3, 20 or 4;
 - (ii) a T cell-sensitizing signal-transducing peptide; and
 - 20 (iii) a translocation peptide of 34-112 amino acid residues in length, comprising the amino acid sequence that is at least 90% identical to SEQ ID NO: 3, 20 or 4; and
 - (c) an antigen of a pathogen, located at the C-terminus of the protein transduction domain; wherein:

the T cell-sensitizing signal-transducing peptide has 28-53 amino acid residues in length and comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 31, in which Xaa⁸ is I or L; Xaa¹⁰ is V, F or A, Xaa¹¹ is M or L, Xaa¹⁷ is L or I; and

the APC-binding domain or the CD91 receptor-binding domain is free of the amino acid sequence of Pseudomonas exotoxin A (PE) binding domain I if the protein transduction domain is the translocation peptide (biii).

2. The fusion protein of claim 1, wherein the APC-binding domain or the CD91 receptor-binding domain is a polypeptide comprising an amino acid sequence that is at least 90% identical to the sequence selected from the group consisting of SEQ ID NOs: 5, 9, 6, 7, and 8.
3. The fusion protein of claim 1, further comprising an endoplasmic reticulum retention sequence located at the C-terminus of the fusion protein.
4. The fusion protein of claim 3, wherein the endoplasmic reticulum retention sequence comprises the amino acid sequence of Lys -Asp-Glu-Leu (SEQ ID NO: 14).
5. The fusion protein of claim 1, wherein the fusion protein is free of an endoplasmic reticulum retention sequence at C-terminus thereof if the antigen contains 10 or more epitopes.
6. The fusion protein of claim 1, wherein the protein transduction domain is the fusion polypeptide (bi).
7. The fusion protein of claim 1, wherein the protein transduction domain is the T cell-sensitizing signal-transducing peptide (bii).
8. The fusion protein of claim 7, further comprising an additional linker between the protein transduction domain and the antigen, the additional linker comprising SEQ ID NO: 15.
9. The fusion protein of claim 1, wherein the protein transduction domain is the translocation peptide (biii).
10. The fusion protein of claim 9, further comprising an additional linker between the APC-binding domain or the CD91 receptor-binding domain and the translocation peptide, the additional linker comprising SEQ ID NO: 15.
11. The fusion protein of claim 1, wherein the protein transduction domain comprises the sequence of SEQ ID NO: 30.
12. A vaccine composition comprising:
 - (a) a therapeutically effective amount of the fusion protein of claim 1; and
 - (b) an adjuvant.
13. The fusion protein of claim 1, wherein the APC-binding domain or the CD91 receptor-binding domain is a polypeptide comprising an amino acid sequence selected from the group consisting of SEQ ID NOs: 5, 9, 6, 7, and 8.

14. The fusion protein of claim 1, wherein the T cell sensitizing signal-transducing peptide comprises an amino acid sequence that is at least 90% identical to SEQ ID NO: 1 or 2.
15. The fusion protein of claim 14, wherein the T cell sensitizing signal-transducing peptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 1 and 2.
- 5 16. The fusion protein of claim 1, wherein the translocation peptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO: 3, 20, and 4.
17. A fusion protein or a vaccine composition as claimed in any one of the preceding claims, for use in inducing enhanced pathogen antigen-specific T cell responses.
18. A fusion protein or a vaccine composition as claimed in any one of the preceding claims, for use
10 in killing a disease cell that presents an antigen via class I MHC molecules on the cell membrane of the disease cell.
19. The fusion protein or the vaccine composition for use as claimed in claim 18, wherein the disease cell is a cancer cell.
20. A fusion protein or a vaccine composition as claimed in any one of the preceding claims, for use
15 in preventing, treating infection caused by a pathogen, and/or minimizing symptoms caused by the infection.

FIG. 1

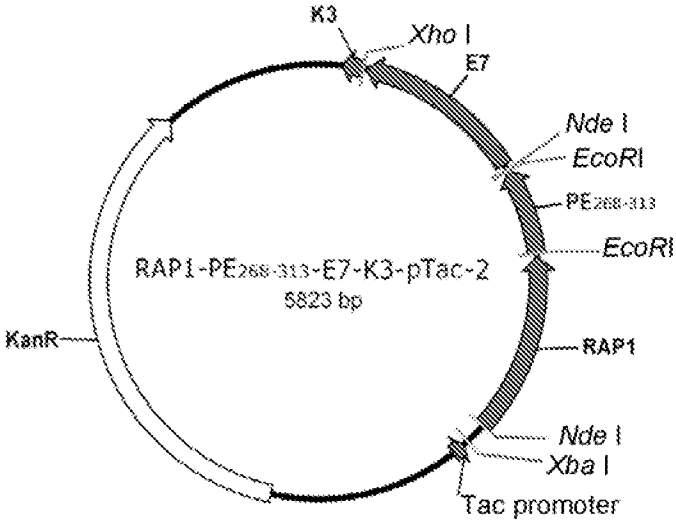


FIG. 3

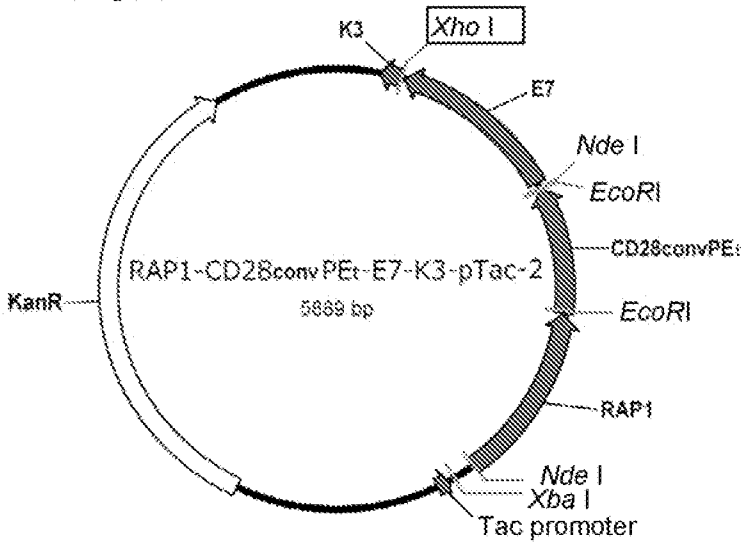


FIG. 4

RAP1-CD28convPE_t-E7-K3 Protein

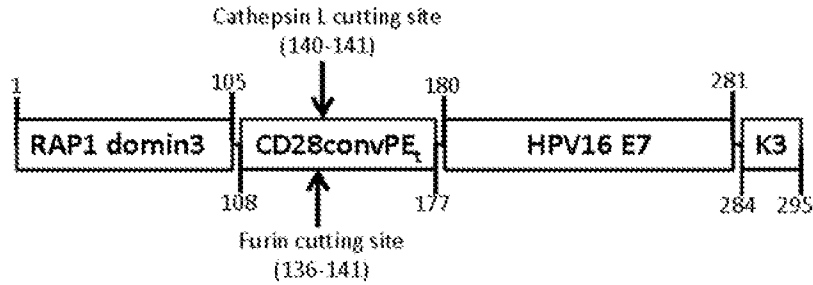
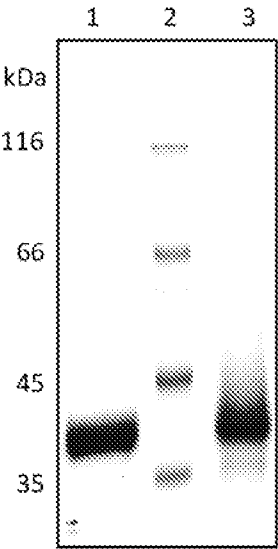


FIG. 2

RAP1-PE₂₆₈₋₃₁₃-E7-K3



Lane 1: RAP1-PE₂₆₈₋₃₁₃-E7-K3 (reduced)
Lane 2: Molecular Marker
Lane 3: RAP1-PE₂₆₈₋₃₁₃-E7-K3 (non-reduced)

FIG. 5A

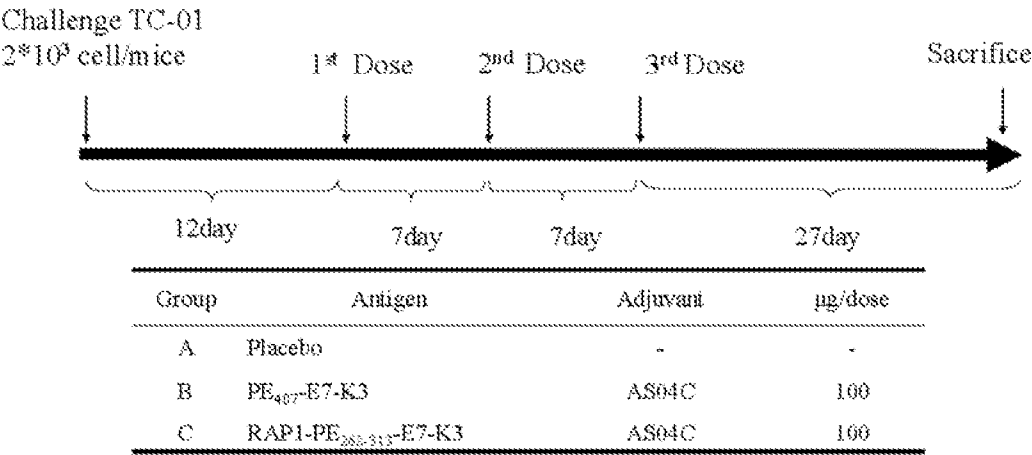
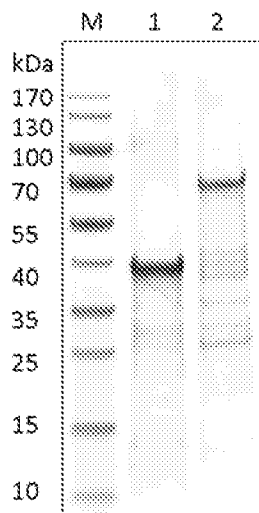


FIG. 5B

1: RAP1-PE₂₆₈₋₃₁₃-E7-K32: PE₄₀₇-E7-K3

M: Molecular weight marker

FIG. 5C

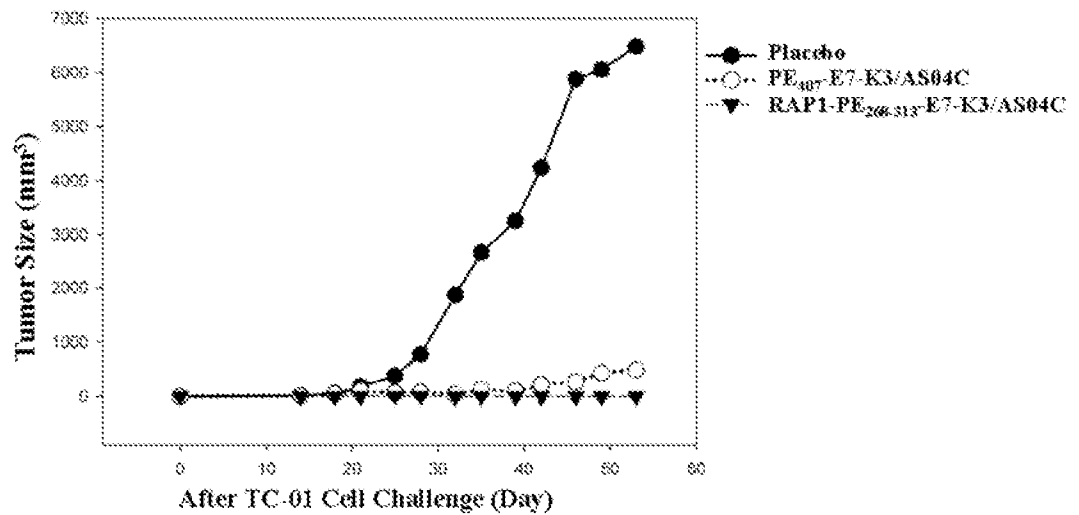


FIG. 5D

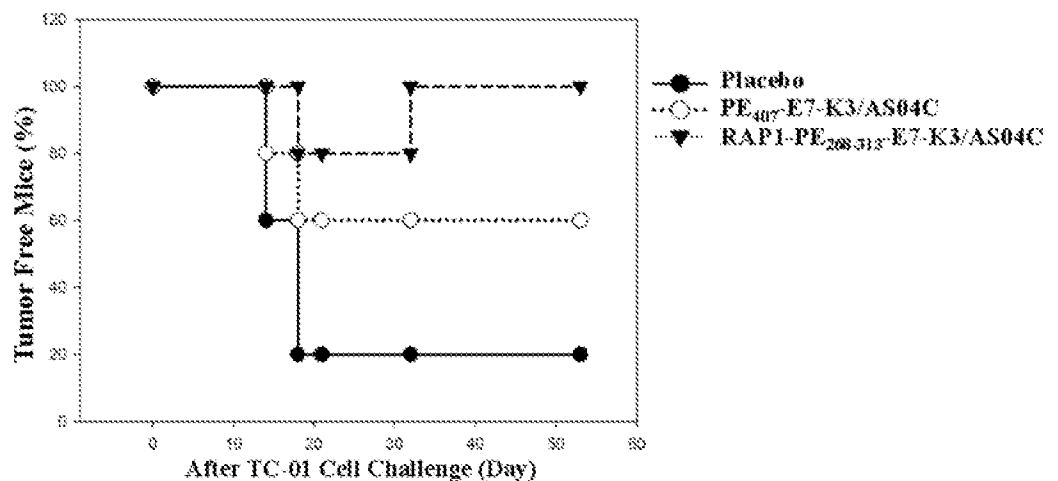


FIG. 6A

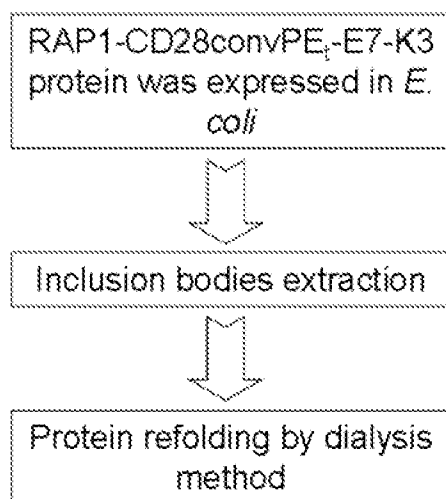
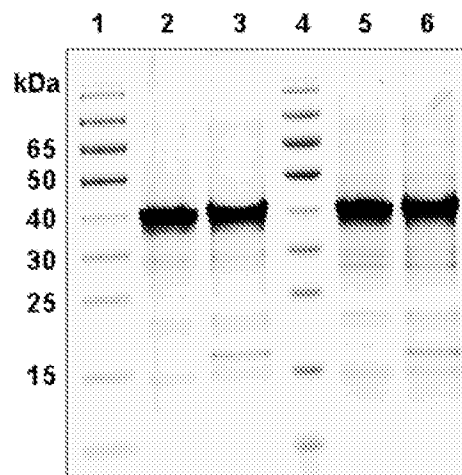


FIG. 6B



Lane 1, 4: Molecular weight marker

Lane 2: RAP1-CD28convPE_E-E7-K3 (mouse CD28conv, reduced)Lane 3: RAP1-CD28convPE_E-E7-K3 (mouse CD28conv, non-reduced)Lane 5: RAP1-CD28convPE_E-E7-K3 (human CD28conv, reduced)Lane 6: RAP1-CD28convPE_E-E7-K3 (human CD28conv, non-reduced)

3/10

FIG. 7

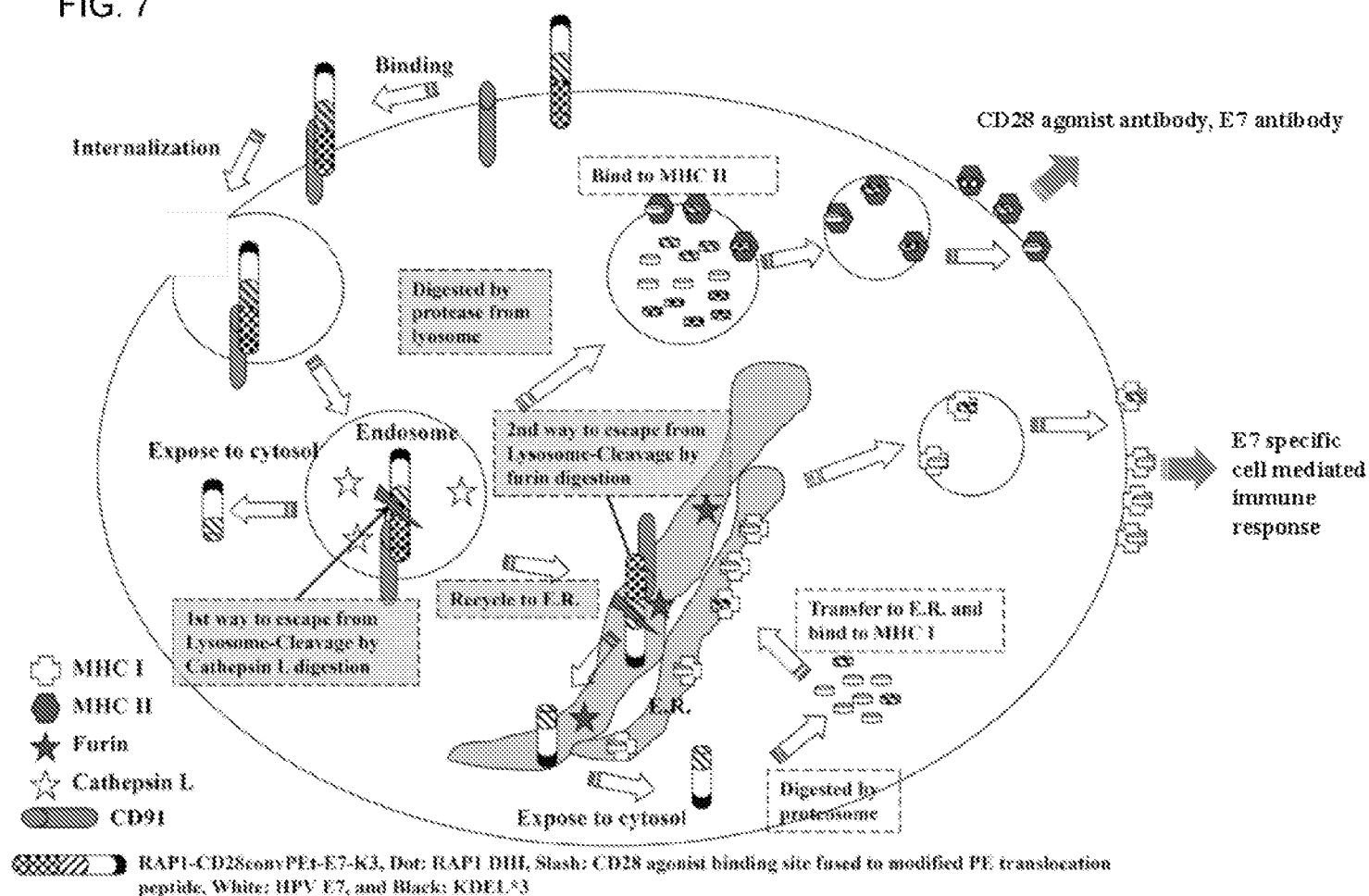


FIG. 8

	101	133
CD28_HUMAN	(100)	ONLYVNQTDIYFCKIEVMYPPPYLDNEKSNGTI
CD28_RAT	(101)	WNLVDNHTDIYFCKIEVMYPPPYLDNEKSNGTI
CD28_MOUSE	(101)	WNLHVNHTDIYFCKIEFMYPYPPPYLDNERSNGTI
CD28-Oryctolagus cuniculus (Rabbit)	(101)	KNLYVNQTDIYFCKIEVMYPPPYLDNEKSNGTI
CD28-Sus scrofa (Pig)	(101)	RNLHVNQTDIYFCKIEVLYPPPYLDNEKSNGTI
CD28-Bos taurus (Bovine)	(99)	QDLYVNQTDIYFCKLEVLYPPPYLDNEKSNGTI
CD28-Ovis aries (Sheep)	(99)	QDLYVNQTDIYFCKLEVLYPPPYLDNEKSNGTI
CD28-Canis lupus familiaris (Dog)	(101)	RNLFVNQTDIYFCKIEVMYPPPYLDNEKSNGTI
CD28-Equus caballus (Horse)	(100)	WNLVNQTDIYFCKIEVMYPPPYLDNEKSNGTI
CD28-Meleagris gallopavo (Turkey)	(99)	RNMTASQTDIYFCKIEAMYPYPPPYLDNEKSNGTI
Consensus	(101)	NLYVNQTDIYFCKIEVMYPPPYLDNEKSNGTI

↑
Critical position for agonist antibody recognizing

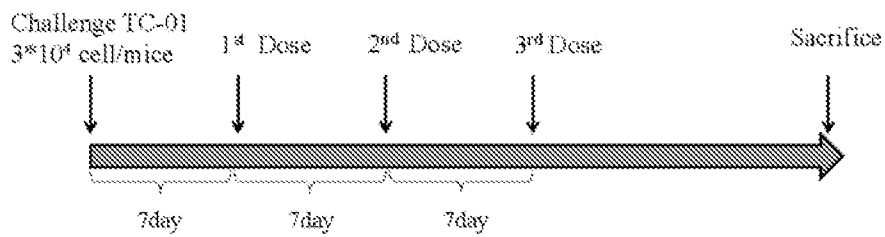


FIG. 9A

Group	Antigen	Adjuvant	µg/dose
A	Placebo	-	-
B	PE ₄₀₇ -E7-K3	GPI-0100	100
C	RAP1-CD28convPE ₄ -E7-K3	GPI-0100	100
D	PE ₄₀₇ -E7-K3	Aluminum Phosphate	100
E	RAP1-CD28convPE ₄ -E7-K3	Aluminum Phosphate	100

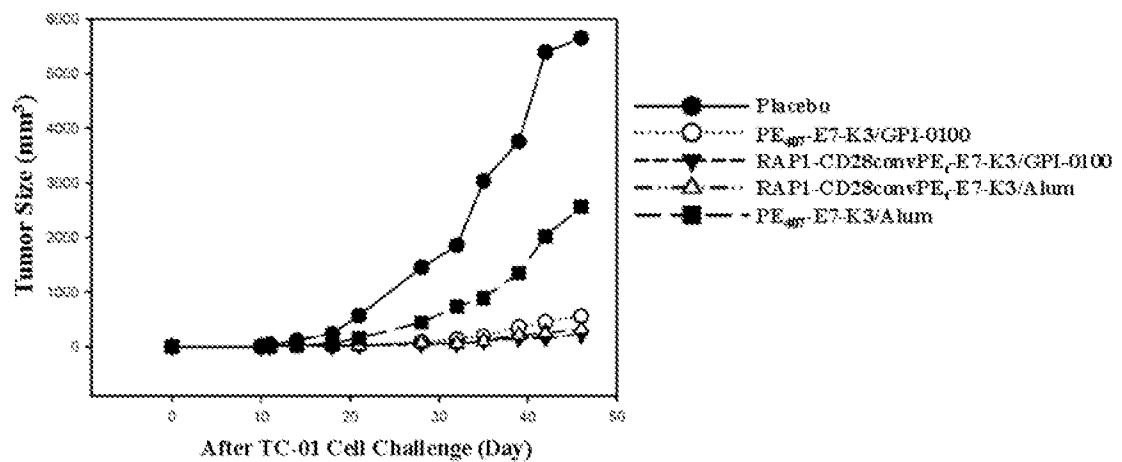


FIG. 9B

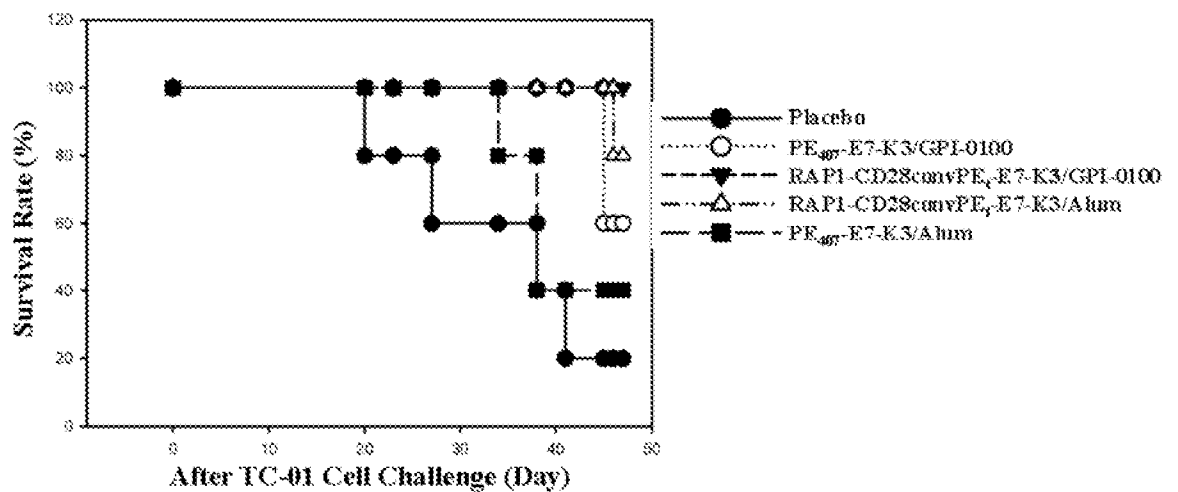


FIG. 9C

FIG. 10

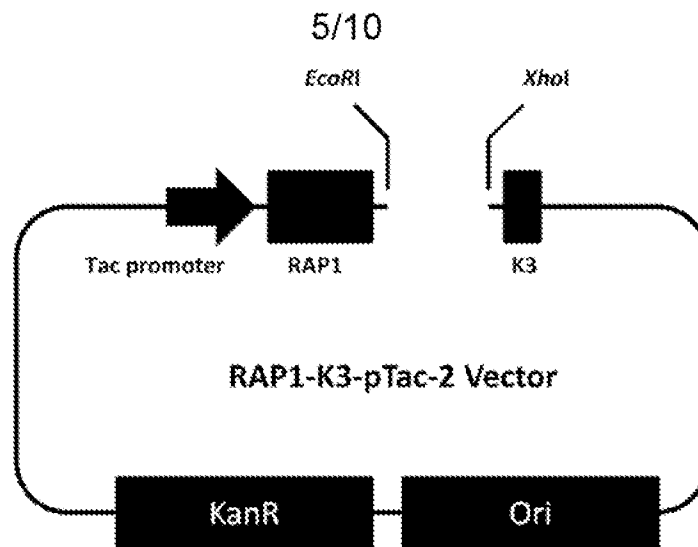


FIG. 11

1. PE ₂₆₈₋₃₁₃ -E7	EcoRI	Linker	PE _i	HPV16 E7	XhoI
2. CD28convPE _i -E7	EcoRI	CD28 epitope	PE _i	HPV16 E7	XhoI
3. CD28convPE _i -E7 ₁₈	EcoRI	CD28 epitope	PE _i	HPV18 E7	XhoI
4. HCVcore	EcoRI			HCV core protein	XhoI
5. CD28convPE _i -HCVcore	EcoRI	CD28 epitope	PE _i	HCV core protein	XhoI
6. CD28conv-HCVcore	EcoRI	CD28 epitope		HCV core protein	XhoI
7. HBx	EcoRI			HBV X protein	XhoI
8. CD28convPE _i -HBx	EcoRI	CD28 epitope	PE _i	HBV X protein	XhoI
9. CD28conv-HBx	EcoRI	CD28 epitope		HBV X protein	XhoI
10. HBx _{stop}	EcoRI			HBV X protein	stop XhoI
11. CD28convPE _i -HBx _{stop}	EcoRI	CD28 epitope	PE _i	HBV X protein	stop XhoI
12. CD28conv-HBx _{stop}	EcoRI	CD28 epitope		HBV X protein	stop XhoI
13. PCV2 _{ORF2}	EcoRI			PCV2 _{ORF2}	XhoI
14. PE ₂₆₈₋₃₁₃ -PCV2 _{ORF2}	EcoRI	Linker	PE _i	PCV2 _{ORF2}	XhoI
15. CD28convPE _i -PCV2 _{ORF2}	EcoRI	CD28 epitope	PE _i	PCV2 _{ORF2}	XhoI
16. PE ₂₆₈₋₃₁₃ -DGD	EcoRI	Linker	PE _i	PRRSV ORF7 ₆₄₋₁₂₃	XhoI
17. CD28convPE _i -DGD	EcoRI	CD28 epitope	PE _i	PRRSV ORF7 ₆₄₋₁₂₃	XhoI
18. PE ₂₆₈₋₃₁₃ -M12	EcoRI	Linker	PE _i	PRRSV ORF1b ₁₀₄₆₋₁₂₁₀	XhoI
19. CD28convPE _i -M12	EcoRI	CD28 epitope	PE _i	PRRSV ORF1b ₁₀₄₆₋₁₂₁₀	XhoI
20. PE ₂₆₈₋₃₁₃ -PQAB	EcoRI	Linker	PE _i	American strain: ORF62-26+ORF531-63	XhoI
21. CD28convPE _i -PQAB	EcoRI	CD28 epitope	PE _i	American strain: ORF62-26+ORF531-63	XhoI
22. PE ₂₆₈₋₃₁₃ -RSAB	EcoRI	Linker	PE _i	European strain: ORF62-28+ORF531-64	XhoI
23. CD28convPE _i -RSAB	EcoRI	CD28 epitope	PE _i	European strain: ORF62-28+ORF531-64	XhoI

6/10

FIG. 12A

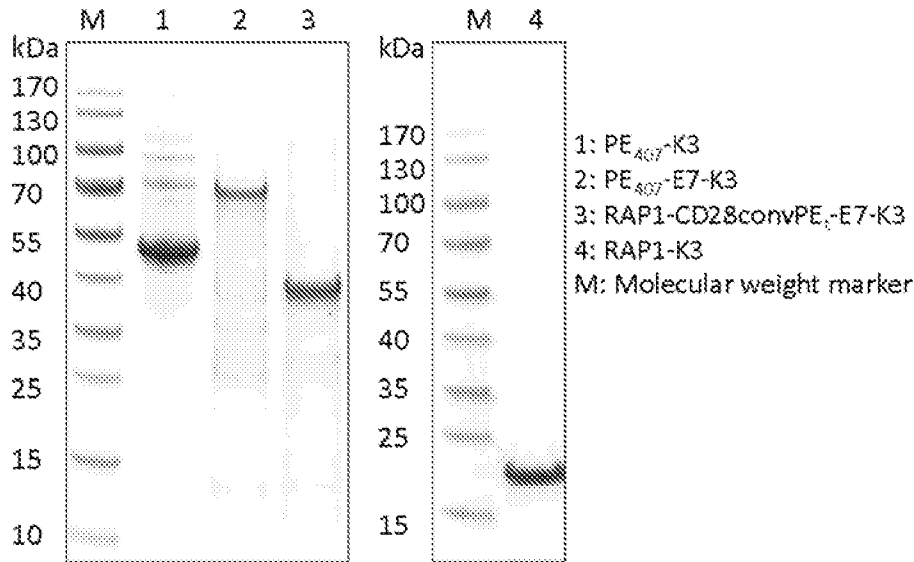


FIG. 12B

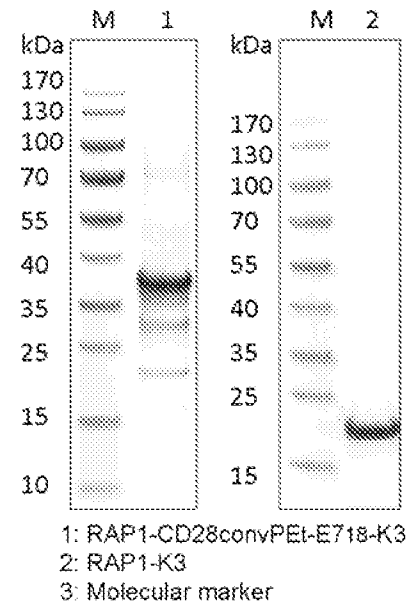


FIG. 12C

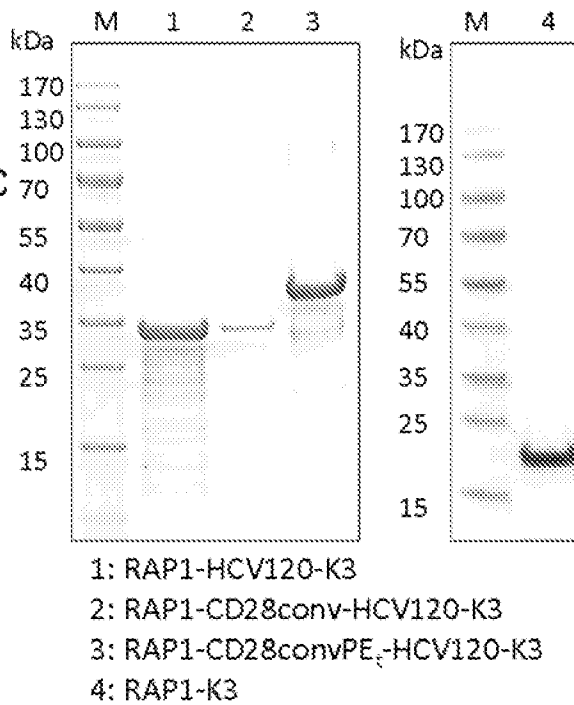


FIG. 12D

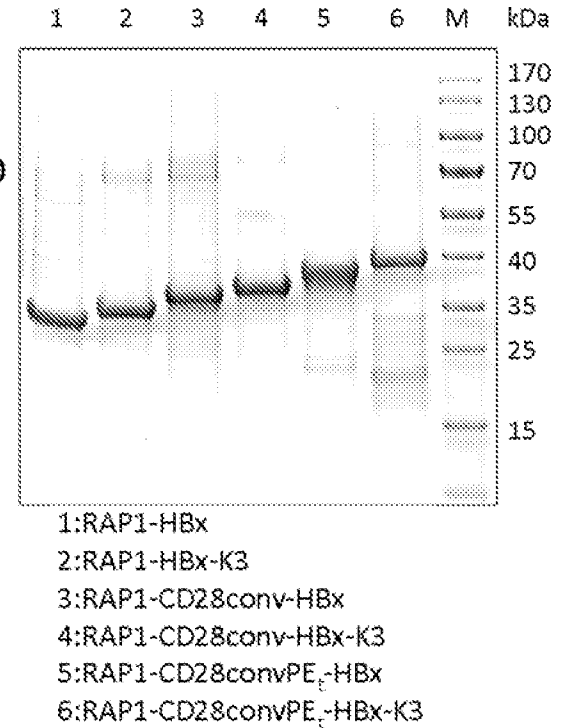


FIG. 12E

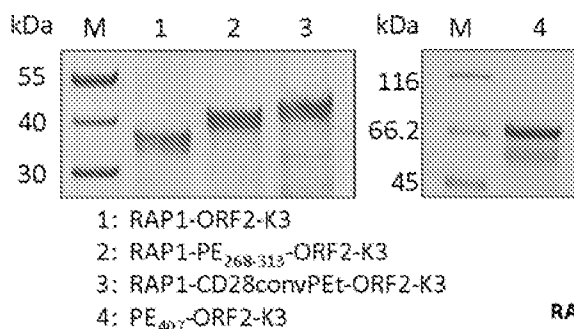
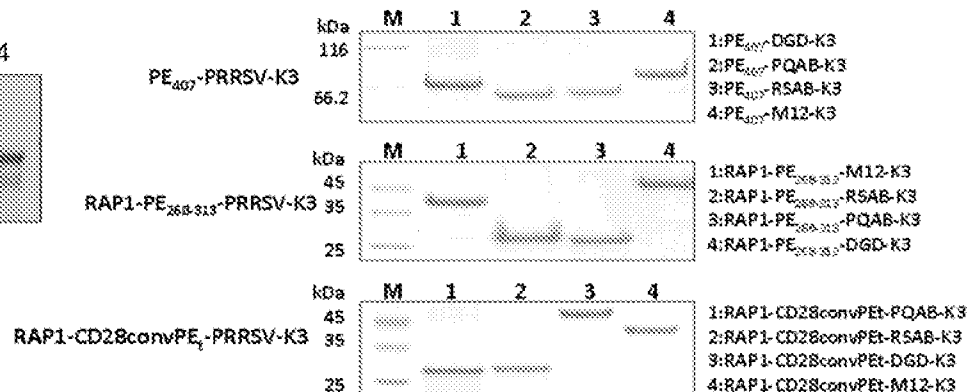


FIG. 12F



7/10

FIG. 13A

Group	Sub-group	Vaccine	Protein absorbent	µg/dose
HPV16 E7	A	Placebo	Aluminum Phosphate	-
	B	PE ₄₀₇ -K3	Aluminum Phosphate	100
	C	PE ₄₀₇ -E7-K3	Aluminum Phosphate	100
	D	RAP1-K3	Aluminum Phosphate	100
	E	RAP1-CD28convPE _t -E7-K3	Aluminum Phosphate	100
HPV18 E7	A	Placebo	Aluminum Phosphate	-
	B	RAP1-K3	Aluminum Phosphate	100
	C	RAP1-CD28convPE _t -E7 ₁₈ -K3	Aluminum Phosphate	100
HCV core	A	Placebo	Aluminum Phosphate	-
	B	RAP1-K3	Aluminum Phosphate	200
	C	RAP1-HCVcore-K3	Aluminum Phosphate	200
	D	RAP1-CD28conv-HCVcore-K3	Aluminum Phosphate	200
	E	RAP1-CD28convPE _t -HCVcore-K3	Aluminum Phosphate	200
HBV HBx	A	Placebo	Aluminum Phosphate	-
	B	RAP1-HBx	Aluminum Phosphate	100
	C	RAP1-HBx-K3	Aluminum Phosphate	100
	D	RAP1-CD28conv-HBx	Aluminum Phosphate	100
	E	RAP1-CD28conv-HBx-K3	Aluminum Phosphate	100
	F	RAP1-CD28convPE _t -HBx	Aluminum Phosphate	100
	G	RAP1-CD28convPE _t -HBx-K3	Aluminum Phosphate	100
PCV2 ORF2	A	Placebo	Aluminum Phosphate	-
	B	PE ₄₀₇ -PCV2-K3	Aluminum Phosphate	40
	C	RAP1-PCV2-K3	Aluminum Phosphate	40
	D	RAP1-PE ₂₆₈₋₃₁₃ -PCV2-K3	Aluminum Phosphate	40
	E	RAP1-CD28convPE _t -PCV2-K3	Aluminum Phosphate	40
PRRSV antigen	A	Placebo	Aluminum Phosphate	-
	B	PE ₄₀₇ -PRRSV-K3 ^a	Aluminum Phosphate	30
	C	RAP1-PE ₂₆₈₋₃₁₃ -PRRSV-K3 ^b	Aluminum Phosphate	30
	D	RAP1-CD28convPE _t -PRRSV-K3 ^c	Aluminum Phosphate	30

a. Each dose contains a mixture of PE₄₀₇-DGD-K3 (12 µg), PE₄₀₇-M12-K3 (6 µg), PE₄₀₇-PQAB-K3 (6 µg), and PE₄₀₇-RSAB-K3 (6 µg).

b. Each dose contains a mixture of RAP1-PE₂₆₈₋₃₁₃-DGD-K3 (12 µg), RAP1-PE₂₆₈₋₃₁₃-M12-K3 (6 µg), RAP1-PE₂₆₈₋₃₁₃-PQAB-K3 (6 µg), and RAP1-PE₂₆₈₋₃₁₃-RSAB-K3 (6 µg).

c. Each dose contains a mixture of RAP1-CD28convPE_t-DGD-K3 (12 µg), RAP1-CD28convPE_t-M12-K3 (6 µg), RAP1-CD28convPE_t-PQAB-K3 (6 µg), and RAP1-CD28convPE_t-RSAB-K3 (6 µg).

8/10

FIG. 13B

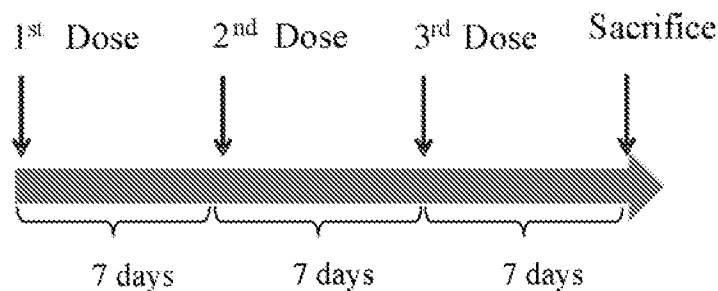


FIG. 14A

		<i>Ex vivo</i> T Cell Immunogenicity (5×10^5 splenocytes)			
		CD3+/CD4+/IFN γ + T cell counts		CD3+/CD8+/IFN γ + T cell counts	
Group	Vaccine	Without E7 Antigen Stimulation	With E7 Antigen Stimulation	Without E7 Antigen Stimulation	With E7 Antigen Stimulation
A	Placebo	13	14	24	33
B	PE ₄₀₇ -K3	21	26	16	25
C	PE ₄₀₇ -E7-K3	18	40	29	34
D	RAP1-K3	8	17	26	28
E	RAP1-CD28convPE ₄₀₇ -E7-K3	10	198	20	232

FIG. 14B

		<i>Ex vivo</i> T Cell Immunogenicity (1.5×10^6 splenocytes)			
		CD3+/CD4+/IFN γ + T cell counts		CD3+/CD8+/IFN γ + T cell counts	
Group	Vaccine	Without E7 ₁₈ Antigen Stimulation	With E7 ₁₈ Antigen Stimulation	Without E7 ₁₈ Antigen Stimulation	With E7 ₁₈ Antigen Stimulation
A	Placebo	2	6	2	0
B	RAP1-K3	2	12	5	36
C	RAP1-CD28convPE ₄₀₇ -E7 ₁₈ -K3	12	614	49	328

FIG. 14C

		<i>Ex vivo</i> T Cell Immunogenicity (1.5×10^6 splenocytes)			
		CD3+/CD4+/IFN γ + T cell counts		CD3+/CD8+/IFN γ + T cell counts	
Group	Vaccine	Without HCVcore Antigen Stimulation	With HCVcore Antigen Stimulation	Without HCVcore Antigen Stimulation	With HCVcore Antigen Stimulation
A	Placebo	13	8	24	34
B	RAP1-K3	8	48	28	33
C	RAP1-HCVcore-K3	13	100	15	54
D	RAP1-CD28conv-HCVcore-K3	11	158	18	260
E	RAP1-CD28convPET-HCVcore-K3	13	236	23	521

FIG. 14D

		<i>Ex vivo</i> T Cell Immunogenicity (5×10^5 splenocytes)			
		CD3+/CD4+/IFN γ + T cell counts		CD3+/CD8+/IFN γ + T cell counts	
Group	Vaccine	Without HBx Antigen Stimulation	With HBx Antigen Stimulation	Without HBx Antigen Stimulation	With HBx Antigen Stimulation
A	Placebo	3	5	1	1
B	RAP1-HBx	6	18	3	9
C	RAP1-HBx-K3	2	10	6	4
D	RAP1-CD28conv-HBx	1	23	6	8
E	RAP1-CD28conv-HBx-K3	9	16	1	18
F	RAP1-CD28convPET-HBx	4	34	4	74
G	RAP1-CD28convPET-HBx-K3	2	191	0	120

9/10

FIG. 15A

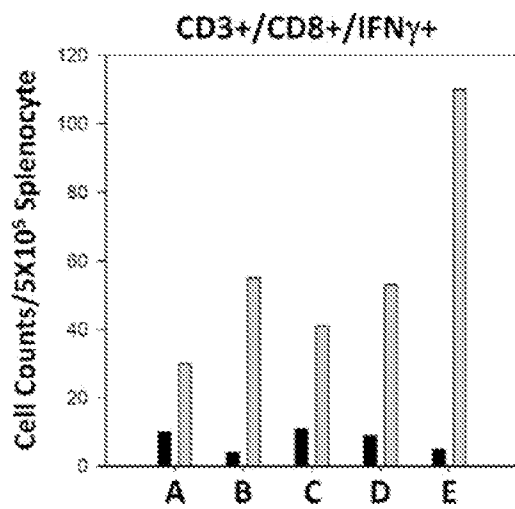
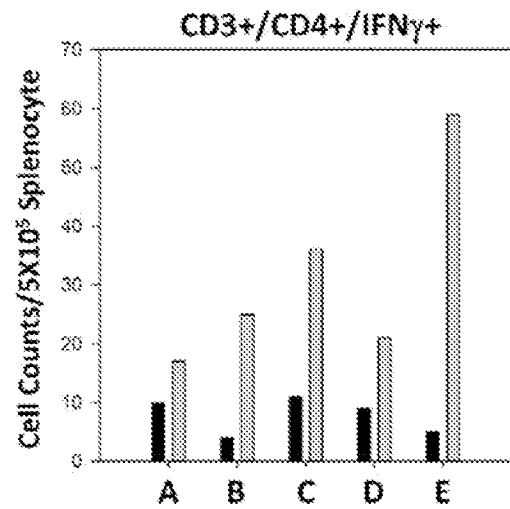


FIG. 15B



■ Without PCV2 ORF2 antigen stimulation
 ▨ With PCV2 ORF2 antigen stimulation

FIG. 15C

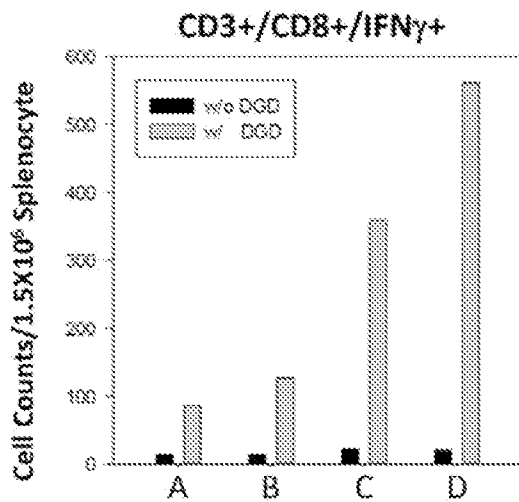


FIG. 15D

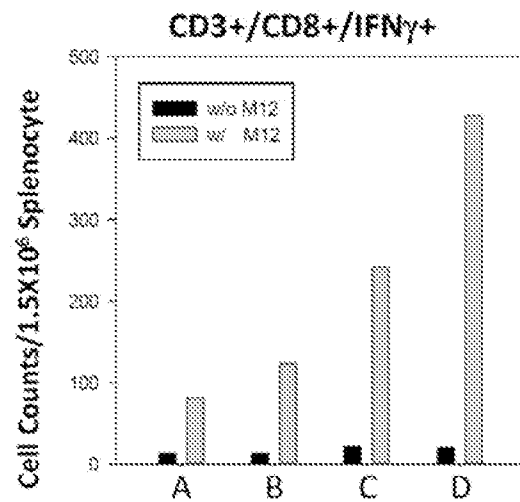


FIG. 15E

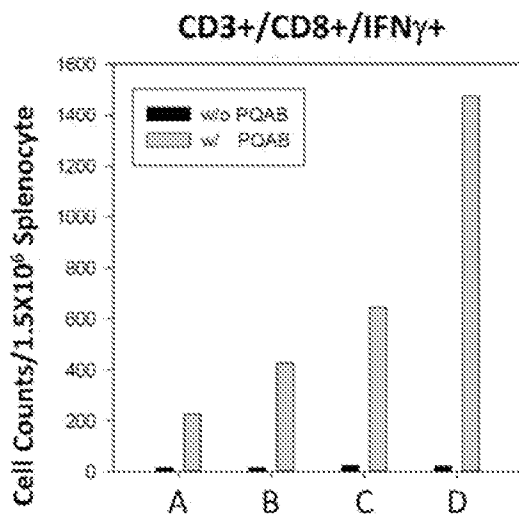


FIG. 15F

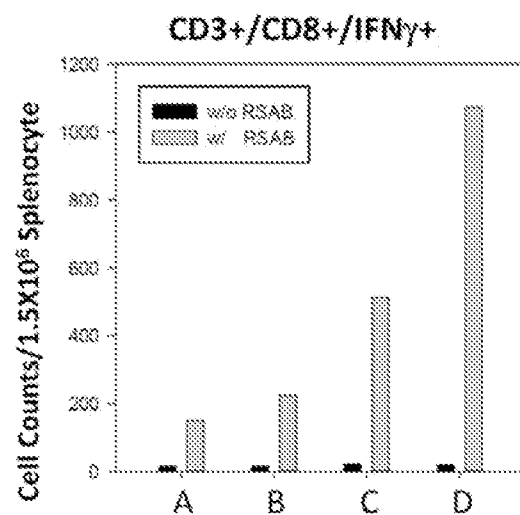


FIG. 15G

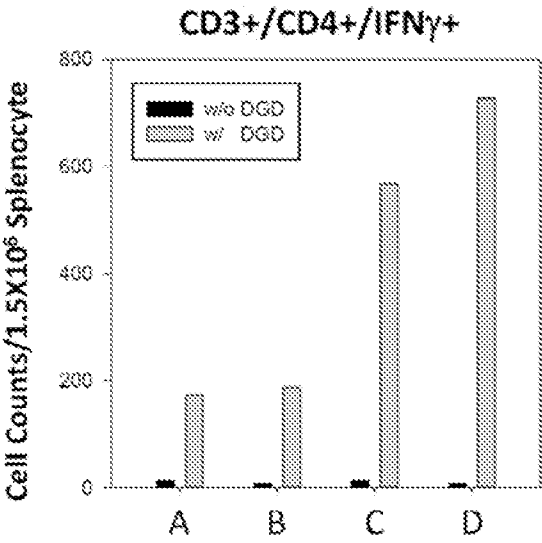


FIG. 15H

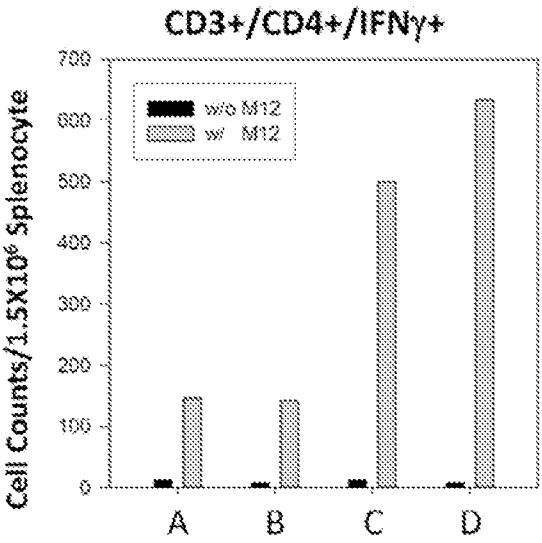


FIG. 15I

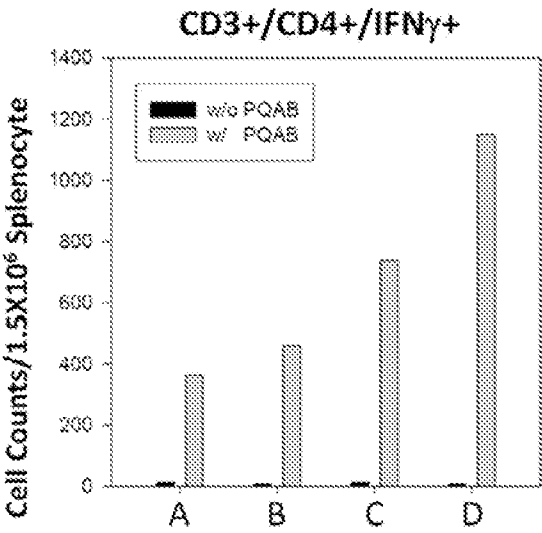
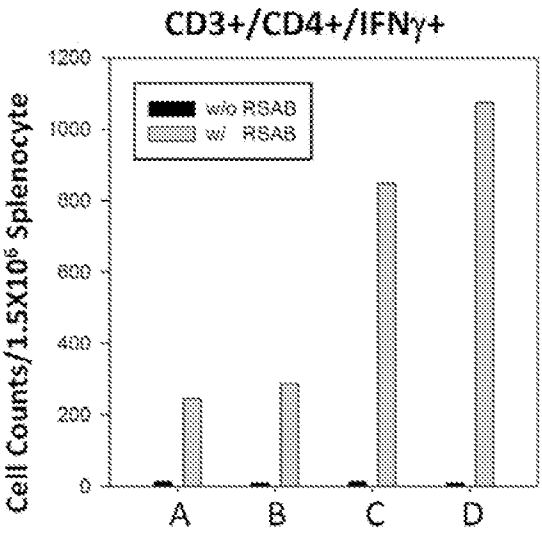


FIG. 15J



INTERNATIONAL SEARCH REPORT

2013/07/27 55 US 04-2014

International application No.

PCT/US 13/72753

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 39/385, A61K 39/00, A61K 38/00 (2014.01)

USPC - 424/194.1, 424/184.1, 514/19.3

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)
USPC- 424/194.1, 424/184.1, 514/19.3Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC- 536/23.4, 424/185.1, 424/188.1, 424/192.1, 424/277.1, 424/136.1, 530/387.3, 530/350, 435/375, 514/9.3, 514.44RElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST(PGPB,USPT,USOC,EPAB,JPAB); PatBase, Google/Patents/Scholar: Fusion, conjugate, LRPAP1, LRP1, low density lipoprotein receptor-related protein 1, A2MR, Alpha-2-macroglobulin receptor, Alpha-2-macroglobulin receptor-associated protein, Alpha-2-MRAP, A2MRAP, APOER, Apolipoprotein E receptor, APR, CD91, LRP. GenCore 6.4.1: SEQ ID No:20.9,15.1,2

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y -- A	Takemoto, et al. Enhanced generation of cytotoxic T lymphocytes by heat shock protein 70 fusion proteins harboring both CD8(+) T cell and CD4(+) T cell epitopes. Mol Pharm. 2010, 7(5):1715-23; Abstract, pg 1718, col 1 and Fig 1 and its legend	1-4, 9, 10, 12, 13, 16, 17/(1-4,9,10,12,13,16) ----- 6-8, 11, 14, 15, 17/(6-8,11,14,15)
Y -- A	US 2009/0214570 A1 (Mrsny, et al.) 27 August 2009 (27.08.2009) claims 1, 28, 32, 39; para [0014]-[0016], [0021], [0132], [0134], [0255], SEQ ID NO 34, amino acids 28-61; SEQ ID NO 21	1-5, 9, 10, 12, 13, 16, 17/(1-5,9,10,12,16) ----- 6-8, 11, 14, 15, 17/(6-8,11,14,15)
Y	WO 2000/49041 A1 (SHINBARA, et al.) 24 August 2000 (24.08.2000) SEQ ID NO:4	2, 13, 17/(2,13)
Y	Chiang, et al. Novel Synthetic Bipartite Carrier Protein for Developing Glycotope-Based Vaccines. Vaccine ePub 22 October 2012, 30(52): 7573-7581; Abstract, pg 13 [of the posted document], Fig 2 and its legend	1, 5, 17/(1,5)
Y	Egerer, et al. Secreted antiviral entry inhibitory (SAVE) peptides for gene therapy of HIV infection. Mol Ther. 2011, 19(7):1236-44; pg 1238, col 1 and Fig 2 and its legend	10, 17(10)
Y	Pesu, et al. Proprotein convertase furin is preferentially expressed in T helper 1 cells and regulates interferon gamma. Blood 2006, 108(3):983-5; Abstract	10, 17(10)



Further documents are listed in the continuation of Box C.



* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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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

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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

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document member of the same patent family

Date of the actual completion of the international search

20 March 2014 (20.03.2014)

Date of mailing of the international search report

08 APR 2014

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Facsimile No. 571-273-3201

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300

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INTERNATIONAL SEARCH REPORT

013/072753-08-04-2014

International application No.

PCT/US 13/72753

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Evans, et al. Crystal structure of a soluble CD28-Fab complex. Nat Immunol. 2005, 6(3):271-9; pg 274, Fig 3 and its legend	6-8, 17/(6-8)
A	Boomer, et al. An Enigmatic Tail of CD28 Signaling. Cold Spring Harb Perspect Biol. 2010; 2(8): a002436; pg 12, col 2	6-8, 17/(6-8)
A	US 2003/0158102 A1 (Chen, et al.) 21 August 2003 (21.08.2003) SEQ ID NO:8, aa 28-53	14, 15, 17/(14,15)
A	WO 2004/087196 A2 (KAEMPFER, et al.) 14 October 2004 (14.10.2004) Example 12, Fig 6, amino acids 67-119	14, 15, 17/(14,15)
A	US 2009/0018079 A1 (WELS, et al.) 15 January 2009 (15.01.2009) Abstract, SEQ ID NO:30, amino acids 34-68	11, 17/11
A	Rueckert, et al. Vaccines: from empirical development to rational design. PLoS Pathog. Epub 08 November 2012, 8(11):e1003001; Abstract, pg 3 to pg 4, 1st para	1-17
A	Caminschi, et al. Boosting antibody responses by targeting antigens to dendritic cells. Trends Immunol Trends Immunol. February 2012, 33(2):71-7; Abstract	1
A	Park, et al. Enhanced Induction of T Cell Immunity Using Dendritic Cells Pulsed with HIV Tat and HCMV-pp65 Fusion Protein In Vitro. Immune Netw. 2011; 11(3): 182-189; Abstract	1-17
A	Liu, et al. A novel therapeutic fusion protein vaccine by two different families of heat shock proteins linked with HPV16 E7 generates potent antitumor immunity and antiangiogenesis. Vaccine 2008, 26(10):1387-96; Abstract	1-17
A	Pawaria, et al. CD91-dependent programming of T-helper cell responses following heat shock protein immunization. Nat Commun. 2011, 2:521; Abstract	1-17
A	Binder, et al. CD91-Dependent Modulation of Immune Responses by Heat Shock Proteins: A Role in Autoimmunity. Autoimmune Dis. Epub 19 Nov 2012, 863041; Abstract	1-17
A	Cruz, et al. Enhancing immunogenicity and cross-reactivity of HIV-1 antigens by in vivo targeting to dendritic cells. Nanomedicine (Lond) October 2012, 7(10):1591-610; pg 1597, Table 1; pg 1598 to pg 1601	1-17
A	US 2012/0021996 A1 (Srivastava, et al.) 26 January 2012 (26.01.2012) para [0016]-[0024]	1-17
A	US 2006/0217298 A1 (Srivastava, et al.) 28 September 2006 (28.09.2006) [0159]-[0176]	1-17
A	US 2012/0263748 A1 (Wu, et al.) 18 October 2012 (18.10.2012) Abstract	1-17
A	US 2008/0132450 A1 (Lee, et al.) 05 June 2008 (05.06.2008) Abstract, claims 1-14; para [0032], FIG. 1B	1-17
A	US 2012/0065385 A1 (Pardoll, et al.) 15 March 2012 (15.03.2012) para [0273]-[0275]	1-17
A	US 2011/0028403 A1 (LE POOLE, et al.) 03 February 2011 (03.02.2011) para [0013]	1-17
A	US 2012/0213811 A1 (Liao, et al.) 23 August 2012 (23.08.2012) Abstract	1-17
A	US 2004/0247617 A1 (Liao, et al.) 09 December 2004 (09.12.2004) claims 1, 8	1-17
A	US 2005/0232932 A1 (Laus, et al.) 20 October 2005 (20.10.2005) claims 1-9, para [0087]	1-17
A	US 2009/0285771 A1 (Mrsny) 19 November 2009 (19.11.2009) SEQ ID NO: 38	1-17
A	WO 2012/036746 A1 (Mrsny, et al.) 22 March 2012 (22.03.2012) pg 3, 2nd para to pg 4, 2nd para	1-17

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 13/72753

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 18-20
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims: it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.