VACCINE COMPOSITIONS AGAINST PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME AND PORCINE CIRCOVIRUS ASSOCIATED DISEASES

FIG. 2A

Abstract: A fusion protein comprising an antigen-presenting cell (APC)-binding domain or a CD91 receptor-binding domain, a translocation peptide, a fusion antigen, an endoplasmic reticulum retention sequence, and optionally a nuclear export signal is disclosed. The fusion antigen comprises a porcine reproductive and respiratory syndrome virus (PRRSV) ORF7 antigen, a PRRSV ORF1b antigen, a PRRSV ORF6 antigen, and a PRRSV ORF5 antigen. The fusion protein is useful for inducing antigen-specific cell-mediated and humoral responses.
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VACCINE COMPOSITIONS AGAINST PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME AND PORCINE CIRCOVIRUS ASSOCIATED DISEASES

FIELD OF THE INVENTION

The present invention relates generally to vaccines, and more specifically to subunit vaccines.

BACKGROUND OF THE INVENTION

Viruses that infect immune cells (such as T-cell, B-cell, dendritic cell, monocyte, or macrophage) include porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type II (PCV2), and human immunodeficiency virus (HIV). The immune cells cannot evoke immunization responses but carry the viruses. The animals that have been infected by these viruses can be easily infected by other pathogens. Porcine reproductive and respiratory syndrome virus (PRRSV) results in high losses in animal husbandry every year. Not only swine but ducks can be infected by PRRSV as well. Generally, the animals infected by the virus have no significant symptoms, but the immunity of the infected animals is reduced. This virus infects macrophages (in the alveolar and spleen), brain microglia and monocytes, and can exist in the blood and organs of the infected animals. This leads to a decrease of weight gain and an increase in the death rate due to the secondary infection.

U.S. Patent No. 7,595,054 discloses a fusion antigen used as a subunit vaccine, in which a single antigen moiety selected from a region of ORF1b or a region of ORF7 is fused between a Pseudomonas exotoxin A polypeptide that is devoid of the cytotoxic domain III, i.e., PE (ΔIII), and an endoplasmic reticulum retention sequence.

A vaccine composition named “PRRSFREETM” by Reber Genetics Co. Ltd. comprises four separate PRRS antigens, which are designated as D, M, R, and P, respectively. These four PRRS antigens were respectively expressed by four separate vectors using the design disclosed in U.S. Patent No. 7,595,054, and were found effective in inducing cell-mediated and humoral immune responses in animals.

SUMMARY OF THE INVENTION

In one aspect, the invention relates to a porcine reproductive and respiratory syndrome virus (PRRSV) 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 translocation peptide of 34-112 amino acid residues in length, comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 4, 2, 3, or 6, located at the C-terminus of the APC-binding domain or the CD91 receptor-binding domain;

(c) a fusion antigen comprising:
(i) a porcine reproductive and respiratory syndrome virus (PRRSV) ORF7 antigen;
(ii) a PRRSV ORF1b antigen;
(iii) a PRRSV ORF6 antigen; and
(iv) a PRRSV ORF5 antigen;

(d) an endoplasmic reticulum retention sequence, located at the C-terminus of the fusion protein; and
(e) optionally a nuclear export signal comprising the amino acid sequence of SEQ ID NO: 13, located between the antigens and the endoplasmic reticulum retention sequence or between the translocation peptide and the antigens;

wherein the fusion antigen does not comprise full-length ORF7, ORF6, ORF5, and ORF1b protein sequences.

In one embodiment of the invention, the ORF7 or ORF1b antigen is located N-terminal to the ORF6 antigen, and the ORF5 antigen is located C-terminal to the ORF6 antigen.

In another embodiment of the invention, the ORF6 antigen is located N-terminal to the ORF5 antigen without a bridge or a linker between the ORF6 and ORF5 antigens.

In another embodiment of the invention, the fusion antigen comprises two tandem repeats of the ORF7 antigen.

In another embodiment of the invention, the ORF5 antigen is located C-terminal to the ORF6 antigen.

In another embodiment of the invention, the ORF6 antigen comprises the N-terminal portion amino acid sequence of the PRRSV ORF6 and the ORF5 antigen comprises the N-terminal portion amino acid sequence of the PRRSV ORF5, and the fusion antigen does not comprise the C-terminal portion amino acid sequences of the ORF6 and ORF5.

In another embodiment of the invention, the ORF1b antigen comprises the C-terminal portion amino acid sequence of ORF1b NSP 10 and the N-terminal portion amino acid sequence of ORF1b NSP 11, and the fusion antigen is devoid of the N-terminal and C-terminal portion amino acid sequences of the ORF1b.

In another 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 SEQ ID NO: 1, 8, 9, 10, 11, or 32.

In another embodiment of the invention, the endoplasmic reticulum retention sequence comprises the amino acid sequence KDEL (SEQ ID NO: 15) without a tandem repeat of the amino acids KDEL with the proviso that the nuclear export signal is present.

In another 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 SEQ
ID NO: 1 or 32.

In another embodiment of the invention, the nuclear export signal and the ER retention sequence forms a fusion peptide with an amino acid sequence that is at least 90% identical to SEQ ID NO: 12.

5 In another embodiment of the invention, the endoplasmic reticulum retention sequence comprises the amino acid sequence of SEQ ID NO: 16, 17, 18, or 19 with the proviso that the nuclear export signal is not present.

In another 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 SEQ ID NO: 8.

10 In another aspect, the invention relates to a composition comprising:
   (i) the PRRSV fusion protein of the invention; and
   (ii) a porcine circovirus type 2 (PCV2) 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 translocation peptide of 34-112 amino acid residues in length, comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 4, 2, 3, or 6, located at the C-terminus of the APC-binding domain or the CD91 receptor-binding domain; and
15 (c) a PCV2 ORF2 antigen;
   (d) an endoplasmic reticulum retention sequence, located at the C-terminus of the fusion protein; and
   (e) a nuclear export signal comprising the amino acid sequence of SEQ ID NO: 13, located between the antigens and the endoplasmic reticulum retention sequence or between the translocation peptide and the antigens;

20 wherein the PCV2 ORF2 antigen comprises the C-terminal portion amino acid sequence of PCV2 ORF 2 protein, and the PCV2 fusion protein does not comprise the N-terminal portion amino acid sequence of the PCV2 ORF2 protein.

In another embodiment of the invention, 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.

In another embodiment of the invention, the translocation peptide has 34-46 amino acid residues in length.

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

30 Further in another aspect, the invention relates to a method for inducing antigen-specific
cell-mediated and humoral responses, comprising administering a composition comprising a therapeutically effective amount of the fusion protein of the invention to a subject in need thereof, and thereby inducing antigen-specific cell-mediated and humoral responses.

Yet in another aspect, the invention relates to use of a composition comprising a therapeutically effective amount of the fusion protein of the invention or the composition of the invention in the manufacture of a medicament for inducing antigen-specific cell-mediated and humoral responses in a subject in need thereof. The invention also relates to a composition comprising a therapeutically effective amount of the fusion protein of the invention or the composition of the invention for use in inducing antigen-specific cell-mediated and humoral responses in a subject in need thereof.

Alternatively, the invention relates to a method for inducing antigen-specific cell-mediated and humoral responses, comprising administering the composition of the invention to a subject in need thereof, and thereby inducing antigen-specific cell-mediated and humoral responses.

The fusion antigen of the invention comprises neutralization and protective epitopes on ORF7, ORF6, ORF5, and ORF1b without comprising full-length ORF7, ORF6, ORF5, and ORF1b protein sequence.

The ORF7 antigen comprises the amino acid sequence of SEQ ID NO: 33, 22, or 23.

The ORF1b antigen comprises the amino acid sequence of the C-terminal portion of ORF1b NSP 10 and the N-terminal portion of ORF1b NSP 11 and is devoid of the N-terminal and C-terminal portions of ORF1b. That is, the fusion antigen comprises the amino acid sequence of the C-terminal portion of ORF1b NSP 10 and the N-terminal portion of ORF1b NSP 11 and does not comprise the amino acid sequence of the N-terminal and C-terminal portions of ORF1b.

The ORF6 antigen comprises the N-terminal portion amino acid sequence of the PRRSV ORF6 and the ORF5 antigen comprises the N-terminal portion amino acid sequence of the PRRSV ORF5, and the fusion antigen does not comprise the C-terminal portion amino acid sequences of ORF6 and ORF5. In other words, the ORF6 antigen is selected from the N-terminal portion amino acid sequence of the PRRSV ORF6, and the ORF5 antigen is selected from the N-terminal portion amino acid sequence of the PRRSV ORF5.

In another embodiment of the invention, the N-terminal portion amino acid sequence of the PRRSV ORF6 is SEQ ID NO: 34, and the N-terminal portion amino acid sequence of the PRRSV ORF5 is 35.

Further in another embodiment of the invention, the N-terminal portion amino acid sequence of the PRRSV ORF6 is SEQ ID NO: 36, and the N-terminal portion amino acid sequence of the PRRSV ORF5 is 37. The ORF1b antigen comprises the C-terminal portion amino acid sequence of ORF1b NSP 10 and the N-terminal portion amino acid sequence of ORF1b NSP 11, and the fusion antigen is devoid of the N-terminal and C-terminal portion amino acid sequences of the ORF1b. In
one embodiment of the invention, the ORF1b antigen has less than 200 amino acid residues in length and comprises the amino acid sequence of SEQ ID NO: 25. In another embodiment of the invention, the ORF1b antigen comprises an amino acid sequence from amino acid residue 1046 to amino acid residue 1210 of the PRRSV ORF1b. In one embodiment of the invention, the C-terminal amino acid of the SEQ ID NO: 13 is alanine.

These and other aspects will become apparent from the following description of the preferred embodiment taken in conjunction with the following drawings. 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. 1A is a schematic drawing showing a full-length Pseudomonas aeruginosa exotoxin A (PE), and partial fragment of PE.

FIGS. 1B-C show vector maps.

FIG. 1D is a schematic drawing showing four separate plasmids that are used for preparation of a vaccine composition that is named as PRRSFREE. The vaccine composition PRRSFREE comprises four separate, individual PE fusion proteins. Each individual PE fusion protein in the PRRSFREE vaccine composition comprises a PE(3III) fragment (PE_{407}), a single antigen moiety (designated as M, P, R, or D), and an endoplasmic retention sequence (K3). The term “D” or “DGD” represents an antigen from PRRSV nucleoprotein ORF7. The term “R” or “RSAB” represents a fusion antigen of PRRSV ORF6/Membrane protein and ORF5/ major envelop protein without a bridge/linker sequence in-between. The term “M” or “M12” represents an antigen from PRRSV ORF1b, and is an artificial fusion antigen of PRRSV nonstructural proteins NSP 10 and NSP 11. The term “P” or “PQAB” represents a fusion antigen of PRRSV ORF6/Membrane protein and ORF5/ major envelop protein without a bridge/linker sequence in-between. The term " PE(3III) " represents a PE fragment without the cytotoxic domain III. The term “PE_{407}” represents a *Pseudomonas exotoxin A* (PE) polypeptide from amino acid 1 to amino acid 407.

FIG. 1E is a schematic drawing showing a plasmid that is used for preparation of a PE fusion protein called PE-DRMP-NESK or PRRSFREE 4-in-one. The PE-DRMP-NESK fusion protein comprises a PE(3III) fragment (PE_{313}), a single fusion polypeptide comprising four antigen moieties (designated as DRMP), a nuclear export signal (NES), and an endoplasmic retention sequence (K).

FIG. 1F is a schematic drawing showing a plasmid encoding a fusion protein comprising a PE(3III) fragment (PE_{313}), a single antigen moiety (designated as PCV2), a nuclear export signal (NES), and an endoplasmic retention sequence (K).
FIG. 2A is a graph showing antigen-specific cell-mediated immune (CMI) responses in mice immunized with PBS, or the vaccine composition PRRSFREE 4-in-1 or PRRSFREE.

FIG. 2B is a graph showing antigen specific antibody (IgG) responses for mice immunized with PBS or PBS, or the vaccine composition PRRSFREE 4-in-1 or PRRSFREE.

FIG. 3A is a graph showing PRRSFREE antigen specific CMI response for mice immunized with PBS or different PRRS/PCV2 combo vaccines.

FIG. 3B is a graph showing PCV2 ORF2 antigen specific CMI response for mice immunized with PBS or different PRRS/PCV2 ORF2 combo vaccines.

FIG. 4A is a graph showing PRRSFREE antigen specific antibody (IgG) response for mice immunized with PBS or different PRRS/PCV2 combo vaccines.

FIG. 4B is a graph showing PCV2 ORF2 antigen specific antibody (IgG) response for mice immunized with PBS or different PRRS/PCV2 combo vaccines.

FIG. 5 is a graph showing PRRSFREE antigen specific CMI responses in mice immunized with (1) a fusion protein comprising a fusion of two antigens (a fusion of the antigens D and R in PE313-DR-NESK), or (2) a combination of two separate fusion proteins, each fusion protein comprising a fusion of two antigens (a fusion of D and R in PE313-DR-NESK, or a fusion of M and P in PE313-MP-NESK), or (3) a fusion protein comprising a fusion of four antigens (a fusion of D, R, M, and P in PE313-DRMP-NESK).

FIG. 6 is a schematic drawing showing various fusion proteins used for immunizing swine against PRRSV infection.

FIG. 7 is a graph showing IFN-γ secreted by PBMC from vaccinated swine after stimulation with respective PRRSV antigens.

**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.

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 are dendritic cells (DCs), macrophages, which are also CD4+ and are therefore also susceptible to infection by HIV; monocytes, and certain B-cells.

The term “an antigen-presenting cell (APC)-binding domain” refers to a domain (which is a polypeptide) 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: 1 and 8-11. 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 “PE₄” refers to a translocation peptide (or a translocation domain) with 34-112 amino acid residues in length. PE₄ may comprises the amino acid sequence that is at least 90% identical to SEQ ID NO: 2-4 and 6. For example, the amino acid sequence of PE₄ may be a fragment of a.a. 280
- a.a. 313 (SEQ ID NO: 4), a.a. 268 - a.a. 313 (SEQ ID NO: 3), a.a. 253 - a.a. 313 (SEQ ID NO: 2), or a.a. 253 - a.a. 364 (SEQ ID NO: 6) of PE. That is, the amino acid sequence of PE may contain any region of the PE domain II (a.a. 253 to a.a. 364; SEQ ID NO: 6) as long as it comprises a.a. 280-a.a. 313 (SEQ ID NO: 4) essential sequence (i.e., the essential fragment).

The PE407 (SEQ ID NO: 7) is described in prior patent (US 7,335,361 B2) as PE(AIII).

The term “minimum translocation peptide” refers to PE253-313 (SEQ ID NO: 2), which can translocate an antigen into the cytoplasm of a target cell.

The term “an endoplasmic reticulum (ER) retention sequence” refers to a peptide whose function is to assist translocation of an antigen from the cytoplasm into ER and retains the antigen in the lumen of the ER. An ER retention sequence comprises the sequence of Lys Asp Glu Leu (KDEL; SEQ ID NO: 15) or RDEL. An ER retention sequence may comprise the sequence KDEL, RDEL, KDELKDELKDEL (K3; SEQ ID NO: 16), KKDLRDELKDEL (K3; SEQ ID NO: 17), KKDELKDELKDEL (K3; SEQ ID NO: 18), or KKDELRLKDEL (K3; SEQ ID NO: 19).

A nuclear export signal (NES) refers to a short amino acid sequence of 4 hydrophobic residues in a protein that targets it for export from the cell nucleus to the cytoplasm through the nuclear pore complex using nuclear transport. The NES is recognized and bound by exportins. The most common spacing of the hydrophobic residues to be L_xKL_xxL_x (SEQ ID NO, 13), where “L” is leucine, “K” is lysine and “x” is any naturally occurring amino acid. For example, an artificial NES may comprise the sequence Leu Gln Lys Lys Leu Glu Glu Leu Glu Leu Ala (LQKKLEEELELA; SEQ ID NO: 14).

The term “NESK” refers to a fusion peptide of a NES and an ER retention signal (i.e., a NES fused to an ER retention signal). It is an artificial peptide possessing the function of a nuclear export signal (NES) and an ER retention sequence. Thus, it can export an antigen from the cell nucleus to the cytoplasm through the nuclear pore complex, and assist translocation of an antigen from the cytoplasm to ER and retain the antigen in the lumen of the ER. For example, the amino acid sequence of NESK may be LQKKLEEELELAKDEL (SEQ ID NO: 12).

Subunit vaccines are vaccines that use only part of the disease-causing virus. This strategy is used most often when one part of the virus is responsible for creating disease. The part responsible for creating disease is a protein, which we call the antigen.

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. 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.

An epitope is a part of antigen. A protective epitope means when the epitope combines with an
antibody, it helps in the functioning of the antibody instead of going against it.

The presence of neutralizing or neutralization epitopes is the structural basis of prophylactic vaccines. Neutralizing epitopes are critical for viral cell attachment/entry.

As used herein, “a porcine reproductive and respiratory syndrome virus (PRRSV) ORF7 antigen” is a peptide that is selected from a portion of PRRSV ORF7 and contains protective epitopes.

As used herein, “a PRRSV ORF1b antigen” is a peptide that is selected from a portion of PRRSV ORF1b and contains protective epitopes.

As used herein, “a PRRSV ORF6 antigen” is a peptide that is selected from a portion of PRRSV ORF6 and contains protective epitopes.

As used herein, “a PRRSV ORF5 antigen” is a peptide that is selected from a portion of PRRSV ORF5 and contains protective epitopes.

The term “PRRSFREE” refers to a vaccine composition comprising the four fusion proteins PE$_{407}$-M-K3, PE$_{407}$-P-K3, PE$_{407}$-R-K3, and PE$_{407}$-D-K3.

The terms “M12” and “M” are interchangeable. The term “M12” as used herein refers to a fusion antigen from fusion of PRRSV NSP 10 (C-terminal domain sequence) and NSP 11 (N-terminal domain sequence).

The terms “PQAB” and “P” are interchangeable. The term “P” as used herein refers to a fusion antigen from fusion of the N-terminal portion of PRRSV ORF6 and the N-terminal portion of ORF5 without a bridge/linker sequence between the ORF6 and ORF5 sequences.

The terms “RSAB” and “R” are interchangeable. The term “R” as used herein refers to a fusion antigen from fusion of the N-terminal portion of PRRSV ORF6 and the N-terminal portion of ORF5 without a bridge/linker sequence between the ORF6 and ORF5 sequences.

The terms “DGD” and “D” are interchangeable. The term “D” as used herein refers to an antigen comprising two repeats of the C-terminal portion of PRRSV ORF7.

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 the route of administration, excipient usage, and the possibility of co-usage with other therapeutic treatment.
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.

Methods

10 **Synthesis of the fusion antigens DRMP and MDPR**

DNA sequences encoding the fusion antigens DRMP (SEQ ID NO: 52), MDPR (SEQ ID NO: 53) and PCV2 ORF2 antigen (SEQ ID NO: 20) were respectively synthesized and further cloned into the plasmids pTAC-2-PE313-NESK or pTAC-2-RAPI-PET268-313-K3. All synthesized sequences were optimized for E.coli growth. Respective forward and reverse primers were used in PCR for DRMP or MDPR DNA amplification. The amplified DNA fragment was digested by EcoRI and XhoI, then was ligated into the indicated vector. The fusion protein PE313-PCV2-NESK was cloned in a similar way.

Table 1 shows the sequences of the forward and reverse primers used for cloning into plasmids. Bold letters indicate EcoRI cutting site; Italic letters indicate Sall cutting site; Italic and bold letters indicate XhoI cutting site; Underlined letters indicate antigen sequence.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>For Cloning DRMP into</td>
<td><strong>gaatccgacctcaccacactttaccecc</strong>&lt;sub&gt;cag&lt;/sub&gt; (SEQ ID NO: 42)</td>
<td><strong>ctcgagaccccatgtaattttagccacag</strong> (SEQ ID NO: 43)</td>
</tr>
<tr>
<td>pTAC-2-PE313-NESK</td>
<td></td>
<td></td>
</tr>
<tr>
<td>For cloning MDPR to</td>
<td><strong>gaattcaataacaaagaagagcgcggggt</strong>&lt;sub&gt;gt&lt;/sub&gt; (SEQ ID NO: 44)</td>
<td><strong>ctcgagaccccattgtttagacag</strong> (SEQ ID NO: 45)</td>
</tr>
<tr>
<td>pTAC-2-PE313-NESK</td>
<td></td>
<td></td>
</tr>
<tr>
<td>For cloning DRMP to</td>
<td><strong>gaatccacacctttacceccgagtga</strong>&lt;sub&gt;gct&lt;/sub&gt; (SEQ ID NO: 46)</td>
<td><strong>ctcgagaccccaatgtttagacag</strong> (SEQ ID NO: 47)</td>
</tr>
<tr>
<td>pTAC-2-RAPI-PET268-313-K3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>For cloning MDPR to</td>
<td><strong>gaatccataacaaagaagcgcggt</strong>&lt;sub&gt;gct&lt;/sub&gt; (SEQ ID NO: 48)</td>
<td><strong>ctcgagaccccaatgtttagacag</strong> (SEQ ID NO: 49)</td>
</tr>
<tr>
<td>pTAC-2-RAPI-PET268-313-K3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>For cloning PCV2 ORF2 to</td>
<td><strong>gaatccgacgaatcttca</strong></td>
<td><strong>ctcgagaccccaatgtttagacag</strong></td>
</tr>
<tr>
<td>pTAC-2-PE313-NESK</td>
<td>(SEQ ID NO: 50)</td>
<td>(SEQ ID NO: 51)</td>
</tr>
</tbody>
</table>
EXAMPLE 1

Construction of Expression Vectors

FIG. 1A shows PE contains 3 domains (I, II, and III). PE_{407} is the region from a.a. 1 to a.a. 407 of PE. PE_{407} does not contain the cytotoxic domain III and thus contains domains I and II. PE_{313} is the region from a.a. 1 to a.a. 313 of PE. Thus, PE_{313} contains only domain Ia and a partial N-terminal region of domain II of PE.

FIGs. 1B-C show constructions of expression vectors, each of which comprises an antigen-presenting cell (APC)-binding domain, a translocation peptide, an antigen, with (bottom panel) or without (top panel) a nuclear export signal (NES); and an endoplasmic reticulum (ER) retention sequence (top panel, K3 or bottom panel, K), the ER retention sequence being located at the C-terminus of the fusion protein. The plasmids pTac-2-PE_{313}-NESK, pTac-2-PE_{407}-K3, pTac-2-RAP1-PE_{268-313}-NESK and pTac-2-RAP1-PE_{268-313}-K3 were generated as follows: The NdeI_{PE_{313}}{(EcoRI, XhoI)}-NESK_{XhoI}, NdeI_{PE_{407}}{(EcoRI, XhoI)}-K3_{XhoI}, NdeI_{RAP1}{(EcoRI)}-PE_{268-313}{(EcoRI, XhoI)}-K3_{XhoI} fragments were synthesized by a PCR method and then ligated into a pUC18 back bond with kanamycin resistance gene to obtain respective plasmids.

A target DNA encoding an antigen or a fusion antigen of a pathogen of interest may then be inserted into the aforementioned plasmids to generate an expression vector for expression of a fusion protein. For example, a DNA fragment encoding an antigen of Porcine Circovirus Type 2 (PCV2) ORF2 (SEQ ID NO: 20) was synthesized and inserted into the plasmids pTac-2-PE_{313}-NESK to generate the expression vector PE_{313}-PCV2-NESK (FIG. 1F).

The following target DNA fragments were synthesized:

(i) a target DNA encoding an antigen comprising two repeats of the C-terminal portion of PRRSV ORF7. The antigen is designated as “DGD” or “D”.

(ii) a target DNA encoding a fusion antigen from fusion of PRRSV NSP 10 (C-terminal domain sequence) and NSP 11 (N-terminal domain sequence). The antigen is designated as “M12” or “M”.

(iii) a target DNA encoding a fusion antigen from fusion of the N-terminal portion of PRRSV ORF6 and the N-terminal portion of ORF5 without a bridge/linker sequence between the ORF6 and ORF5 sequences. The antigen is designated as “RSAB” or “R”.

(iv) a target DNA encoding a fusion antigen from fusion of the N-terminal portion of PRRSV ORF6 and the N-terminal portion of ORF5 without a bridge/linker sequence between the ORF6 and ORF5 sequences. The antigen is designated as “PQAB” or “P”.

The above target DNA fragments were inserted into the plasmid shown in FIG. 1B upper panel to generate fusion proteins PE_{407}-M-K3, PE_{407}-P-K3, PE_{407}-R-K3, and PE_{407}-D-K3, respectively.
A target DNA fragment encoding a fusion antigen comprising all of the four aforementioned antigens D, R, M, and P (such as DRMP, MDPR, etc.) was synthesized and inserted into the plasmids pTac-2-PE313-NESK to generate an expression vector expressing the fusion protein PE-DRMP-NESK (FIG. 1E), which is designates as (also “PRRSFREE 4-in-1”).

EXAMPLE 2
Protein expression

E. coli BL21 cells harboring plasmids for expression of fusion proteins were respectively cultured in Luria Bertani broth containing 25 ppm of kanamycin at 37°C. When the culture reaching early log phase, (A600=0.1 to 0.4), isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added with a final concentration of 0.5 to 2 mM for induction. Cells were harvested after induction after 4 hours and immediately stored at −70°C. The fusion proteins were purified by urea extraction as described previously (Liao et al., 1995. Appl. Microbiol. Biotechnol. 43: 498-507) and then were refolded by dialysis method against 50X volume of TNE buffer (50mM Tris, 50mM NaCl and 1mM EDTA) at 4°C for overnight. The refolded proteins were subjected to SDS-PAGE analyses and quantitative analyses performed using Bradford Protein Assay Kit (Pierce). The results indicated that most of the refolded proteins were monomers under a non-reduced condition, indicating that the fusion proteins refolded easily and were not aggregated.

EXAMPLE 3
PRRSV subunit vaccines immunogenicity assay

Mice were vaccinated with 200 µl PRRSV subunit vaccine containing 30µg/shot of PRRSFREE 4-in-1 or PRRSFREE and ISA206 adjuvant via s.c. injection once a week for 2 weeks. The control group (placebo) was injected with PBS.

All mice were sacrificed 14 days after the last immunization, and the spleens were harvested.

The splenocytes were isolated and cultured in 96-well plate (10^5 cells/100 µl/well) with or without the stimulant recombinant antigen protein at 37°C for 72 hr. Depending on the vaccine used in immunization, the stimulant recombinant antigen protein used was PRRSFREE antigens, PRRSFREE-4-in-one chimeric fusion antigen, or PCV2 ORF2 antigen for detecting antigen-specific cell-mediated immune response. The cell culture supernatant was collected and interferon-gamma (IFN-γ) in the supernatant was determined by IFN-γ Mouse Antibody Pair (Invitrogen).

Depending on the vaccine used for immunization, PRRSFREE antigens, or PRRSFREE 4-in-one fusion antigen, or PCV2 ORF2 antigen was coated in ELISA plates for detecting humoral immune response. After coating, the plates were washed and blocked before adding diluted mouse serum. Then the plates were washed, hybridized with HRP-conjugated secondary antibody
followed by adding TMB substrate. After the reaction was stopped, the result was detected by ELISA reader.

EXAMPLE 4

Cell-mediated immune response (CMI) and Humoral immune response

FIG. 2A shows that the IFN-γ concentration in the vaccinated groups was higher than that in the control group, indicating that a CMI response was induced upon vaccinations. Furthermore, the IFN-γ concentration of the group receiving PRRSFREE 4-in-1 vaccine was dramatically higher than that in the PRRSFREE-treated group. The result demonstrates that PRRSFREE 4-in-1 vaccine, which was composed of one single fused antigen, can surprisingly induce a stronger CMI response than the PRRSFREE vaccines composed of four separate antigens.

FIG. 2B shows vaccine-immunized groups had higher antigen-specific antibody titers than the control group. Mice vaccinated with the PRRSFREE 4-in-1 vaccine had a higher antibody titer than the group immunized with the PRRSFREE vaccine. The result shows that PRRSFREE 4-in-1 can induce a stronger humoral immune response than the PRRSFREE vaccine.

The data in FIGs. 2A-B indicate that PRRSFREE 4-in-1, which contains a fusion protein comprising one single fusion antigen with fusion of D, M, P, and R antigens, can elicit a stronger cellular and humoral immune responses than the PRRSFREE vaccine, which contains four individual, separate antigens (i.e., the four antigens M, M, P, R are not fused) with each antigen in a respective fusion protein.

EXAMPLE 5

Combination Vaccines with porcine circovirus type 2 (PCV2) ORF2 subunit vaccine

Mice were vaccinated with PBS, PRRSFREE 4-in-one plus PE-PCV2-NESK, or PRRSFREE plus PE-PCV2-NESK combo vaccines according to the immunization schedule as described above. The PRRSFREE 4-in-one plus PE-PCV2-NESK combo vaccine contains PE-DRMP-NESK (FIG. 1D) and PE-PCV2-NESK (FIG. 1F) fusion proteins. The PRRSFREE plus PE-PCV2-NESK combo vaccine contains 5 separate fusion proteins: (1) PE-DGD-K3, PE-M12-K3, PE-PQAB-K3, PE-RSAB-K3 (FIG. 1D), and PE-PCV2-NESK (FIG. 1F).

EXAMPLE 6

Combination Vaccines with PCV2 ORF2 subunit vaccine

FIG. 3A shows PRRSV antigen-specific (PRRSFREE 4-in-1 fusion antigen, and PRRSFREE antigens) and FIG. 3B shows PCV2-ORF2-specific CMI responses. The data indicate that the mice group immunized with the combination of PRRSFREE 4-in-1 fusion antigen and PCV2 ORF2 subunit vaccine showed a stronger CMI response than that in the mice group immunized with the combination of PRRSFREE (4 separate antigens) and PCV2 ORF2 subunit vaccine.

FIG. 4A shows PRRSV antigen-specific antibody responses. An ELISA method was used to
measure antigen-specific antibody titers. For the group treated with the combination of PE-DRMP-NESK and PE-PCV2-NESK (i.e., two fusion proteins), the fusion antigen DRMP was used to measure the antigen-specific antibody titer. For the group treated with the combination of PRRSFREE and PE-PCV2-NESK (i.e., 5 fusion proteins), four antigens D, R, M, and P were used to measure the antigen-specific antibody titer. The data indicate that the mice group immunized with the combination of PRRSFREE 4-in-1 fusion antigen and PCV2 ORF2 subunit vaccine showed a stronger PRRSFREE 4-in-1 fusion antigen-specific humoral response than that in the mice group immunized with the combination of PRRSFREE (4 separate antigens) and PCV2 ORF2 subunit vaccine (FIG. 4A).

FIG. 4B shows PCV2-ORF2 antigen-specific antibody responses. Surprisingly, mice immunized with the combination of PRRSFREE (4 separate antigens) and PCV2 ORF2 subunit vaccine (PE-PCV2-NESK) had a higher PCV2-specific antibody titer than the group immunized with the combination of PRRSFREE 4-in-1 fusion antigen (PE-DRMP-NESK) and PCV2 ORF2 subunit vaccine (PE-PCV2-NESK). The results indicate there was a differential PRRSV antigen-specific and PCV2-specific humoral immune responses between the two PRRSV/PCV2 combo vaccines.

It is clear that both approaches are effective in inducing CMI and humoral immune responses. The PRRSV/PCV2 combo vaccine comprising 2 fusion proteins (PE-DRMP-NESK and PE-PCV2-NESK) shows better efficacy in three out of four immune responses examined. This study demonstrates that PRRSV/PCV2 combo vaccine composed of PRRSV chimeric fusion antigen and PCV2 ORF2 antigen is a better choice than the one composed of 5 individual antigens. Nevertheless, both approaches are useful for inducing immune responses in an animal.

EXAMPLE 7

Fusion of two antigens v. Fusion of 4 antigens

Three groups of 6-weeks-old female C57BL/6 mice (3 mice per group) were injected subcutaneously with (1) 15 µg of PE-DR-NESK protein, (2) a combination of 15 µg PE-DR-NESK and 15 µg of PE-MP-NESK proteins, or (3) 30 µg of PE-DRMP-NESK, in 200 µl of 50% ISA206 at weekly intervals three times. Mice were killed at 1 week after the last immunization, and splenocytes were harvested. Splenocytes were stimulated with 4 PRRSV antigens (M12, DgD, PQAB and RSAB, 2.5 µg/ml of each) for 72 hr, and IFN-γ in the cell-free supernatants of each group were detected using ELISA kit. FIG. 5 shows that mice immunized with PE-DRMP-NESK showed the greatest CMI response among three groups.

EXAMPLE 8

Cell-mediated immunity in swine

Five-weeks-old SPF swine (2-4 swine per group) were injected intramuscularly with one of the following vaccines: (1) PRRSFREE, (2) PE-DRMP-NESK, (3) PE-MDPR-NESK, (4)
RAP1-PE_{268-313}-DRMP-K3, (5) RAP1-PE_{268-313}-MDPR-K3 in 2 ml of 50% ISA206, or (6) PBS as placebo, twice at weekly intervals. FIG. 6 shows designs of these vaccines. The antigen in each injection was 300 µg in 2 ml of 50% ISA206. Peripheral blood mononuclear cells (PBMCs) of vaccinated swine were harvested at 3 week after last immunization. Depending on the vaccine used in immunization, the PBMCs were stimulated with PRRSFREE antigens (M12, DgD, PQAB and RSAB, 2.5 µg/ml of each), PE-DRMP-NEK, PE-MDPR-NEK, RAP1-PE_{268-313}-DRMP-K3, or RAP1-PE_{268-313}-MDPR-K3 for 72 hr, then IFN-γ in the cell-free supernatants of each group were detected using ELISA kit. FIG. 7 shows IFN-γ secreted by PBMC of vaccinated swine after stimulation. It was observed that vaccines comprising fusion antigens could induce higher IFN-γ secretion than placebo group.

EXAMPLE 9

Viremia studies in swine

Five-weeks-old SPF swine (2-4 swine per group) were injected intramuscularly with one of the following vaccines: (1) PRRSFREE, (2) PE-DRMP-NEK, (3) PE-MDPR-NEK, (4) RAP1-PE_{268-313}-DRMP-K3, (5) RAP1-PE_{268-313}-MDPR-K3 in 2 ml of 50% ISA206, or (6) PBS as placebo, twice at weekly intervals. The antigen in each injection was 300 µg in 2 ml of 50% ISA206. The vaccinated swine were intranasally challenged with 2 × 10^5 TCID50 of PRRSV at three weeks after the last immunization. Blood sample were collected weekly. Viral RNA were extracted from the sera and quantified using one-step SyBR Green real-time PCR to determine the levels of viremia. The experimental results indicated that vaccines comprising fusion antigens could reduce the virus load.

Table 2 shows SEQ ID NOs. of peptides used for making various fusion proteins.

Table 2

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<th>Component</th>
<th>SEQ ID NO:</th>
<th>Length</th>
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<td>PE_{253-313} (translocation domain)</td>
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<td>PE_{268-313} (translocation domain)*</td>
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<td>PE_{253-364} (translocation domain)</td>
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<td>PE_{407} (a.a. 1- a.a. 407 of PE)</td>
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<td>NES is LQKKLEELA</td>
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<td>KKDRLVELKDEL (K3)</td>
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<td>PCV2 ORF2 (truncated porcine circovirus type 2 ORF2; aa 42 -aa 233)</td>
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<tr>
<td>Full length PE (Exotoxin A, <em>Pseudomonas aeruginosa</em>)</td>
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<td>DGD (ORF7 antigen with a tandem repeat D)</td>
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<td>D (ORF7 antigen without a tandem repeat D)</td>
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<td>R (RSAB)</td>
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<td>M (M12)***</td>
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<tr>
<td>P (PQAB)****</td>
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<td>N-terminal portion of ORF6 [from a PRRSV isolate in Taiwan]</td>
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<td>N-terminal portion of ORF5 [from a PRRSV isolate in Taiwan]</td>
<td>ASNDSSSHLQLIYNLTCELNGTDWLANKFDWA</td>
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<tr>
<td>----------------------------------------------------------</td>
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<tr>
<td>N-terminal portion of ORF6</td>
<td>MGSLDDFCNDSTAAQKLVLAFSITYTPi</td>
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<tr>
<td>N-terminal portion of ORF5</td>
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<td>PRRSV ORF5, Type 1 (European) PRRSV strain</td>
<td>MRCSHKLGRFLTPHSCFWWLFLCTGLSWSFVAGGSSSTYQYIYNLTCELNGTDWLSNHFDWA VETFVL</td>
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<td>RFTNFIVDDRGRIHRWKSPIVVEKLGKAEVGGDLVTIKHVVLEGVKAKQLTRTSAEQWEA</td>
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<tr>
<td>PRRSV ORF6, Type 1 (European) PRRSV strain</td>
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<td>LHLIFLNCSTFGYMTYVRFQSTNRV</td>
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<td>ALTLGAVVALLWGVYSEFTESWKFVTSRCRLCCLGRRYILAPAHHVESAAGLHISIPASGNRAYAVRKPGLTSVNGTLVPGLRLVGLGGKRAVKGVNNLVKYGR</td>
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<tr>
<td>PRRSV ORF5, Type 2 (North America) PRRSV strain</td>
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<td>PVLTHIVSYGALTTSHFDLTVGLITVSTAGFYHGRYVLSSIYATC</td>
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<td>ALAALICFIVRIKNCMSWRYSCTR</td>
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<td>PRRSV ORF6: Type 2 (North America) PRRSV strain</td>
<td>MGSSLDDFCHDSTAPQKV1AFSITYTPMIYALKVSRGRLLG</td>
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</tbody>
</table>
*: PE268-313 is a.a. 268- a.a. 313 of full length PE; PE313 is a.a. 1- a.a. 313 of full length PE; PE407 is a.a. 1- a.a. 407 of full length PE.

**: The bold letters represents the amino acid sequence of an artificial nuclear exporting signal; the underlined letters represents the amino acid sequence of an endoplasmic reticulum retention signal.

**: M (M12) is a fusion polypeptide prepared by fusion of PRRSV NSP 10 (C-terminal domain sequence) and NSP 11 (N-terminal domain sequence). That is, the polypeptide is derived from the nonstructural proteins ORF1b NSP 10 C-terminal portion and NSP 11 N-terminal portion.

****: P (PQAB) is a polypeptide prepared by fusion of PRRSV ORF6 a.a. 2 - a.a. 26 and ORF5 aa 31- aa 63. See U.S. Patentent No. 7465455. The sequence in regular letters derives from PRRSV ORF6//matrix protein sequence, and the sequence in bold letters derives from PRRSV ORF 5 sequence. The major envelope protein (GP5) encoded by the ORF5 of PRRSV has a critical role in inducing virus neutralizing (VN) antibody and cross protection among different strains of PRRSV. Since there are sequence variations among different strains, the sequences herein are disclosed for illustration purpose.

The present invention is not limited to the particular forms as illustrated, and that all the modifications not departing from the spirit and scope of the present invention are within the scope as defined in the appended claims. 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. Some references, which may include patents, patent applications and various publications, are cited and discussed in the description of this invention. The citation and/or discussion of such references is provided merely to clarify the description of the present invention and is not an admission that any such reference is “prior art” to the invention described herein. All references cited and discussed in this specification are incorporated herein by reference in their entireties and to the same extent as if each reference was individually incorporated by reference.
CLAIMS

What is claimed is:

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 translocation peptide of 34-112 amino acid residues in length, comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 4, 2, 3, or 6, located at the C-terminus of the APC-binding domain or the CD91 receptor-binding domain;
   (c) a fusion antigen comprising:
      (i) a porcine reproductive and respiratory syndrome virus (PRRSV) ORF7 antigen;
      (ii) a PRRSV ORF1b antigen;
      (iii) a PRRSV ORF6 antigen; and
      (iv) a PRRSV ORF5 antigen;
   (d) an endoplasmic reticulum retention sequence, located at the C-terminus of the fusion protein; and
   (e) optionally a nuclear export signal comprising the amino acid sequence of SEQ ID NO: 13, located between the antigens and the endoplasmic reticulum retention sequence or between the translocation peptide and the antigens;

wherein the fusion antigen does not comprise full-length ORF7, ORF6, ORF5, and ORF1b protein sequences.

2. The fusion protein of claim 1, wherein the ORF7 or ORF1b antigen is located N-terminal to the ORF6 antigen, and the ORF5 antigen is located C-terminal to the ORF6 antigen.

3. The fusion protein of claim 1, wherein the fusion antigen comprises two tandem repeats of the ORF7 antigen.

4. The fusion protein of claim 1, wherein the ORF5 antigen is located C-terminal to the ORF6 antigen.

5. The fusion protein of claim 1, wherein the ORF6 antigen comprises the N-terminal portion amino acid sequence of the PRRSV ORF6 and the ORF5 antigen comprises the N-terminal portion amino acid sequence of the PRRSV ORF5, and the fusion antigen does not comprise the C-terminal portion amino acid sequences of the ORF6 and ORF5.
6. The fusion protein of claim 5, wherein the ORF1b antigen comprises the C-terminal portion amino acid sequence of ORF1b NSP 10 and the N-terminal portion amino acid sequence of ORF1b NSP 11, and the fusion antigen is devoid of the N-terminal and C-terminal portion amino acid sequences of the ORF1b.

7. 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 SEQ ID NO: 1, 8, 9, 10, 11, or 32.

8. The fusion protein of claim 1, wherein the translocation peptide has 34-61 amino acid residues in length.

9. The fusion protein of claim 1, wherein the endoplasmic reticulum retention sequence comprises the amino acid sequence KDEL (SEQ ID NO: 15) without a tandem repeat of the amino acids KDEL with the proviso that the nuclear export signal is present.

10. The fusion protein of claim 9, wherein the nuclear export signal and the ER retention sequence forms a fusion peptide with an amino acid sequence that is at least 90% identical to SEQ ID NO: 12.

11. The fusion protein of claim 1, wherein the endoplasmic reticulum retention sequence comprises the amino acid sequence of SEQ ID NO: 16, 17, 18, or 19 with the proviso that the nuclear export signal is not present.

12. The fusion protein of claim 11, 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 SEQ ID NO: 8.

13. A composition comprising:
   (i) the fusion protein of claim 1; and
   (ii) a porcine circovirus type 2 (PCV2) 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 translocation peptide of 34-112 amino acid residues in length, comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 4, 2, 3, or 6, located at the C-terminus of the APC-binding domain or the CD91 receptor-binding domain; and
       (c) a PCV2 ORF2 antigen;
       (d) an endoplasmic reticulum retention sequence, located at the C-terminus of the fusion protein; and
(e) a nuclear export signal comprising the amino acid sequence of SEQ ID NO: 13, located between the antigens and the endoplasmic reticulum retention sequence or between the translocation peptide and the antigens; wherein the PCV2 ORF2 antigen comprises the C-terminal portion amino acid sequence of PCV2 ORF 2 protein, and the PCV2 fusion protein does not comprise the N-terminal portion amino acid sequence of the PCV2 ORF2 protein.

14. The fusion protein of claim 1, wherein the translocation peptide has 34-61 amino acid residues in length.

15. Use of a composition comprising a therapeutically effective amount of the fusion protein of claim 1 or the composition of claim 13 in the manufacture of a medicament for inducing antigen-specific cell-mediated and humoral responses in a subject in need thereof.
A. CLASSIFICATION OF SUBJECT MATTER
IPC(B) - A61K 39/02; A61K 39/10; A61K 39/104 (2016.01)
CPC - A61K 39/12; A61K 2039/552; C07K 14/21 (2016.08)
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)
IPC - A61K 39/02; A61K 39/10; A61K 39/104
CPC - A61K 39/12; A61K 2039/552; C07K 14/21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 424/190.1; 424/204.1; 435/6 (keyword delimited)

Electronic database base consulted during the international search (name of data base and, where practicable, search terms used)
Patbase, Google Patents, PubMed, Google
Search terms used: antigen presenting cell APC PRRSV porcine reproductive respiratory syndrome virus ORF1b ORF6 ORF5 CD91 fusion protein

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>US 8,092,809 B2 (FITZGERALD) 10 January 2012 (10.01.2012) entire document</td>
<td>1, 7-10</td>
</tr>
<tr>
<td>A</td>
<td>US 2012/0251553 A1 (NICCHITTA CHRISTOPHER V et al) 04 October 2012 (04.10.2012)</td>
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<tr>
<td>A</td>
<td>US 8,986,720 B2 (PODACK et al) 03 March 2015 (03.03.2015) entire document</td>
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<td>US 2014/0154285 A1 (THEVAX GENETICS VACCINE CO., LTD.) 05 June 2014 (05.06.2014)</td>
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* Further documents are listed in the continuation of Box C.  
| See patent family annex. |

* Special categories of cited documents:
  "A" document defining the general state of the art which is not considered to be of particular relevance
  "E" earlier application or patent but published on or after the international filing date
  "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  "O" document referring to an oral disclosure, use, exhibition or other means
  "P" document published prior to the international filing date but later than the priority date claimed
  "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
  "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
  "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
  "&" document member of the same patent family

Date of the actual completion of the international search
13 October 2016

Date of mailing of the international search report
27 OCT 2016

Name and mailing address of the ISA/
Mail Stop PCT, Attn: ISA/AUS, Commissioner for Patents
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Form PCT/ISA/210 (second sheet) (January 2015)
1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
   a. ☑ forming part of the international application as filed:
      ☑ in the form of an Annex C/ST.25 text file.
      ☐ on paper or in the form of an image file.
   b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
      ☐ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).
      ☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:
SEQ ID NOs: 1, 4, 12, 25, and 27 were searched.
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.:**
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

see Extra Sheet(s).

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.:**

   1 and 7-10 restricted to a fusion protein of SEQ ID NO: 27; and SEQ ID NOs: 1, 4, 12, and 25.

**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.
Continued from Box No. III Observations where unity of invention is lacking

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees need to be paid.

Group I+: claims 1-15 are drawn to a fusion protein.

The first invention of Group I+ is restricted to a fusion protein, wherein the fusion protein is selected to be SEQ ID NO:27, comprising (a) an antigen-presenting cell (APC)-binding domain or a CD91 receptor-binding domain of SEQ ID NO:1, located at the N-terminus of the fusion protein; (b) a translocation peptide of SEQ ID NO:4, located at the C-terminus of the APC-binding domain or the CD91 receptor-binding domain; (c) a fusion antigen of SEQ ID NO:25 comprising: (i) a porcine reproductive and respiratory syndrome virus (PRRSV) ORF7 antigen, (ii) a PRRSV ORF1b antigen, (iii) a PRRSV ORF6 antigen, and (iv) a PRRSV ORF5 antigen; (d) and (e) an endoplasmic reticulum retention sequence and a nuclear export signal of SEQ ID NO:12. It is believed that claims 1 and 7-10 read on this first named invention and thus these claims will be searched without fee to the extent that they read on a fusion protein of SEQ ID NO: 27.

Applicant is invited to elect additional fusion proteins with corresponding APC/CD91 receptor binding domains with specified SEQ ID NO and/or translocation peptides with specified SEQ ID NO and/or fusion antigens with specified SEQ ID NO and/or endoplasmic reticulum retentions sequences with specified SEQ ID NO and/or nuclear export signal domains with specified SEQ ID NO to be searched in a specific combination by paying additional fee for each set of election. An exemplary election would be a fusion protein, wherein the fusion protein is selected to be SEQ ID NO:28. Additional fusion proteins with specified SEQ ID NO will be searched upon the payment of additional fees. Applicants must specify the claims that read on any additional elected inventions. Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group will result in only the first claimed invention to be searched/examined.

The inventions listed in Groups I+ do not relate to a single general inventive concept under PCT Rule 13.1, because under PCT Rule 13.2 they lack the same or corresponding special technical features for the following reasons:

The Groups I+ fusion proteins do not share a significant structural element responsible for inducing antigen-specific cell-mediated and humoral responses in a subject, requiring the selection of alternatives for the APC/CD91 receptor binding domains, the translocation peptides, and the endoplasmic reticulum retention sequences, where "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 SEQ ID NO: 1, 8, 9, 10, 11, or 32" and "translocation peptide of 34-112 amino acid residues in length, comprising an amino acid sequence that is at least 90% identical to SEQ ID NO: 4, 2, 3, or 6" and "the endoplasmic reticulum retention sequence comprises the amino acid sequence of SEQ ID NO: 16, 17, 18, or 19."

The Groups I+ share the technical features of 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 translocation peptide, located at the C-terminus of the APC-binding domain or the CD91 receptor-binding domain; a fusion antigen comprising: (i) a porcine reproductive and respiratory syndrome virus (PRRSV) ORF7 antigen, (ii) a PRRSV ORF1b antigen, (iii) a PRRSV ORF6 antigen, and (iv) a PRRSV ORF5 antigen; an endoplasmic reticulum retention sequence, located at the C-terminus of the fusion protein; and optionally a nuclear export signal, located between the antigens and the endoplasmic reticulum retention sequence or between the translocation peptide and the antigens; wherein the fusion antigen does not comprise full-length ORF7, ORF6, ORF5, and ORF1b protein sequences. However, these shared technical features do not represent a contribution over the prior art.

Specifically, US 2014/0154285 A1 to The Vax Genetics Vaccine Co. Ltd. discloses (a) an antigen-presenting cell (APC)-binding domain or a CD91 receptor-binding Domain (an antigen-presenting cell 1APC)-binding domain or a CD91 receptor-binding domain,Abstract), located at the N-terminus of the fusion protein (an antigen-presenting cell (APC)-binding domain or a CD91 receptor-binding domain, located at the terminus of the fusion protein, Para. [0000]); (b) a translocation peptide; located at the C-terminus of the APC-binding domain or the CD91 receptor-binding domain (a protein transduction domain, located at the C-terminus of the APC-binding domain or the CD91 receptor-binding domain, Para. [0006]); a fusion antigen comprising: (i) a porcine reproductive and respiratory syndrome virus (PRRSV) ORF7 antigen (DG represents a fusion antigen of PRRSV ORF7 aa 64-aa 123 (boldface), linkor (underlined) and ORF7 aa 64-aa 123 (boldface), Para. [0127]); (ii) a PRRSV ORF1b antigen; (iii) a PRRSV ORF6 antigen and (iv) a PRRSV ORF5 antigen (example, a fusion antigen of PRRSV ORF6 and ORF5 fragments, or a fusion of antigenic proteins from PRRSV and PCV2 pathogens, Para. [0102]); PRRSV ORF1b, See Fig. 10); an endoplasmic reticulum retention sequence, located at the C-terminus of the fusion protein (the fusion protein further comprises an endoplasmic reticulum retention sequence located at the C-terminus of the fusion protein, Para. [0118]); wherein the fusion antigen does not comprise full-length ORF7, ORF6, ORF5, and ORF1b protein sequences (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, Para. [0123]).

The inventions listed in Groups I+ therefore lack unity under Rule 13 because they do not share a same or corresponding special technical features.