

RESPIRATORY SYNCYTIAL VIRUS VACCINE

Field of the Invention

[0001] The present invention generally relates to prophylactic and therapeutic agents against respiratory syncytial virus (RSV), and more particularly to a RSV vaccine comprising a recombinant fusion protein antigen containing phosphoprotein (P) protein.

Background of the Invention

[0002] Respiratory syncytial virus (RSV) is a leading cause for bronchiolitis and severe respiratory disease in infants, young children, immune-compromised and elderly populations worldwide. Natural RSV infection in young children does not elicit long-lasting immunity and individuals remain susceptible to repeated RSV infections throughout life. A formalin-inactivated RSV (FI-RSV) vaccine, tested in infants a half century ago, resulted in enhanced morbidity and two deaths after a subsequent exposure to a natural RSV infection. The infants and children that received the FI-RSV vaccine exhibited a lower level of neutralizing antibodies following a natural infection (Yang and Varga, 2014).

[0003] While much efforts and resources have been devoted to the development of a safe and effective RSV vaccine, there is no licensed RSV vaccine available so far. The researches carried out so far demonstrated that it is a daunting challenge for developing a safe and effective RSV vaccine.

[0004] Therefore, there is an imperative need to explore new ways to develop a safe and effective vaccine against RSV.

Summary of the Invention

[0005] The present invention provides a respiratory syncytial virus (RSV) vaccine comprising a recombinant fusion protein antigen. In one embodiment, the recombinant fusion protein antigen comprises a phosphoprotein (P) moiety, wherein the P moiety is a polypeptide that shares at least 98% identity to the polypeptide represented by SEQ ID NO 2 or SEQ ID NO 4; and a flagellin moiety, wherein the flagellin moiety is a polypeptide that shares at least 98%

identity to the polypeptide represented by SEQ ID NO 8; whereby the P moiety and flagellin moiety are covalently coupled so as to form a linear polypeptide.

[0006] In another embodiment of the RSV vaccine, the P moiety is a polypeptide that shares at least 99% identity to the polypeptide represented by SEQ ID NO 2 or SEQ ID NO 4; and the flagellin moiety is a polypeptide that shares at least 99% identity to the polypeptide represented by SEQ ID NO 8.

[0007] In another embodiment of the RSV vaccine, the P moiety is a polypeptide represented by SEQ ID NO 2 or SEQ ID NO 4; and the flagellin moiety is a polypeptide represented by SEQ ID NO 8.

[0008] In another embodiment of the RSV vaccine, the recombinant fusion protein antigen further comprises a first linker coupling the P moiety and flagellin moiety; wherein the first linker is an amino acid or a peptide of two to fifteen amino acids.

[0009] In another embodiment of the RSV vaccine, the recombinant fusion protein antigen further comprises a purification tag for facilitating the purification of the recombinant fusion protein antigen, wherein the purification tag is disposed at either the N-terminal or C-terminal of the recombinant fusion protein antigen. In further embodiment, the purification tag is composed of six histidine residues. In yet another embodiment, the recombinant fusion protein antigen further comprises a second linker linearly coupling the purification tag with the P moiety or flagellin moiety. In yet further embodiment, the second linker is a cleavable linker that is composed of an amino acid or a sequence of amino acids which can be cleaved chemically or enzymatically at its C-terminal.

[0010] In another embodiment of the RSV vaccine, the recombinant fusion protein antigen is represented by SEQ ID NO 10.

[0011] The objectives and advantages of the invention will become apparent from the following detailed description of preferred embodiments thereof in connection with the accompanying drawings.

Brief Description of the Drawings

[0012] Preferred embodiments according to the present invention will now be described with reference to the Figures, in which like reference numerals denote like elements.

[0013] FIG 1 shows the sequences:

[0014] (a) SEQ ID NO 1, nucleotide sequence of RSV A2 strain P protein;

- [0015] (b) SEQ ID NO 2, amino acid sequence of RSV A2 strain P protein;
- [0016] (c) SEQ ID NO 3, nucleotide sequence of RSV mutant cpt-248 P protein;
- [0017] (d) SEQ ID NO 4, amino acid sequence of RSV mutant cpt-248 P protein;
- [0018] (e) SEQ ID NO 5, nucleotide sequence of recombinant P protein with His tag underlined;
- [0019] (f) SEQ ID NO 6, amino acid sequence of recombinant P protein with His tag, where the RSV mutant cpt-248 P protein as shown as SEQ ID NO 4 is underlined, and the His tag is bolded;
- [0020] (g) SEQ ID NO 7, nucleotide sequence of recombinant flagellin KFD1;
- [0021] (h) SEQ ID NO 8, amino acid sequence of recombinant flagellin KFD1;
- [0022] (i) SEQ ID NO 9, nucleotide sequence of recombinant P-KFD1, where the RSV mutant cpt-248 protein as shown as SEQ ID NO 3 is underlined, the nucleotide sequence of SEQ ID NO 7 is double underlined, and the His tag is bolded;
- [0023] (j) SEQ ID NO 10, amino acid sequence of recombinant P-KFD1, where the RSV mutant cpt-248 protein as shown as SEQ ID NO 4 is underlined, the amino acid sequence of SEQ ID NO 8 is double underlined, and the His tag is bolded.
- [0024] FIG 2(a) shows a schematic diagram of functional blocks in a recombinant P-KFD1 protein in accordance with one embodiment of the present invention; FIG 2(b) shows a western blot of purified P-KFD1 protein.
- [0025] FIG 3 shows a diagram of temporal protocol of immunization and challenge.
- [0026] FIG 4 provides graphs showing the P-specific IgG and IgA antibody titers in serum and vaginal samples from the groups of immunization with P+CTB or P-KFD1.
- [0027] FIG 5 provides graphs showing viral loads in nose or lung after challenge.
- [0028] FIG 6 provides a graph showing the P-specific T cell responses.
- [0029] FIG 7 provides a panel of pictures showing the lung tissues: (a) normal and uninfected; (b) control group immunized with saline and infected with RSV; (c) FI-RSV immunized group infected with RSV; (d) P+CTB immunized group infected with RSV; and (e) P-KFD1 immunized group infected with RSV.
- [0030] FIG 8 is a graph showing the weight changes after challenge.
- [0031] FIG 9 provides graphs: (a) showing the correlation of inspiratory resistance (RI) with different concentrations of methacholine (MCH); and (b) showing the correlation of expiratory resistance (RE) with different concentrations of methacholine (MCH) in different groups after challenge.

[0032] FIG 10 provides graphs showing lymphocyte infiltration into lung tissues at day 8 post challenge: (a) immunocyte numbers; (b) neutrophil frequency in lung; (c) eosinophil frequency in lung; and (d) recruited macrophage frequency in lung.

Detailed Description of the Invention

[0033] The present invention may be understood more readily by reference to the following detailed description of certain embodiments of the invention.

[0034] Throughout this application, where publications are referenced, the disclosures of these publications are hereby incorporated by reference, in their entireties, into this application in order to more fully describe the state of art to which this invention pertains.

[0035] The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, for example, *Molecular Cloning: A Laboratory Manual*, second edition (Sambrook and Russel, 2001); *Current Protocols in Molecular Biology* (F.M. Ausubel et al., eds., 1987).

[0036] RSV has a linear single-stranded RNA genome with 10 genes encoding 11 proteins, including non-structural proteins (NS1 and NS2), large polymerase (L), phosphoprotein (P), nucleocapsid (N), matrix protein (M), surface attachment glycoprotein (G), surface fusion glycoprotein (F), small hydrophic protein (SH), a transcription factor (M2-1) and an accessory protein (M2-2). G and F proteins have been considered the two major protective antigens for eliciting neutralizing antibodies. G protein is heavily glycosylated and involved in viral attachment to host cells, and F protein mediates cell fusion allowing entry of the virus into the cell cytoplasm and formation of syncytia (Yang and Varga, 2014).

[0037] Since RSV F protein is a very important neutralizing antigen to potentially induce mucosal immunity, it has been the focus for development of an RSV vaccine. Based on the recent identification of the very potent neutralizing antigen site in the prefusion F, the next generation of RSV vaccine candidates should include the F protein expressed in the prefusion form (Yang and Varga, 2014).

[0038] Because RSV infection is restricted to the respiratory tract, an RSV vaccine should elicit mucosal immunity at upper and lower respiratory tracts in order to most effectively

prevent RSV reinfection (Yang and Varga, 2014). The efficient induction of mucosal immune responses requires appropriate administration routes and specific adjuvants and/or delivery systems. Intranasal delivery is the most effective route to induce potent and broad mucosal immune responses at multiple mucosal sites as compared to other mucosal delivery routes (Yang and Varga, 2014).

[0039] RSV vaccines have four main categories including inactivated, live-attenuated, gene-based vectors, and subunit. Live-attenuated RSV vaccines administered intranasally, and a subunit RSV postfusion F protein vaccine adjuvanted with alum and delivered intramuscularly have been extensively evaluated in a number of clinical trials in recent years. However, the live-attenuated RSV vaccine induced a response that was weaker in magnitude than that of natural infection due to loss of immunogenicity during the process of attenuation (Yang and Varga, 2014).

[0040] Being delivered usually by systemic immunization, F protein-based RSV vaccines were proven safe in older children and adults, however their immunogenicity was modest and they failed to induce potent mucosal immunity (Groothuis JR, et al. 1998).

[0041] Antigen selections

[0042] VLPs expressing F or G protein could confer protection against RSV challenge in mice, but VLP G vaccination enhanced disease when used alone; one interesting result was that mixed VLP F + VLP G were without vaccine-induced immunopathology even though no plausible explanation was provided (Lee S., et al. 2014).

[0043] Chimpanzee adenovirus and modified vaccinia virus encoding RSV proteins F, N and M2-1 have been tested in phase I clinical trials (Green C.A., 2015).

[0044] VLP containing pre-fusion form F protein was tested in cotton rats for its protection against RSV (Cullen L.M., et al. 2015).

[0045] A peptide corresponding to the viral surface small hydrophobic (SH) ectodomain of HRSV B (CGGGS-NKLSEHKTF SNKTLEQCQMYQINT) chemically conjugated to Hepatitis B core protein virus-like particles (HBc-SHeB) showed reducing viral replication in challenged mice and cotton rats (Schepens, B. et al. 2014).

[0046] Adjuvant selections

[0047] Alum-adjuvanted FI-RSV induced severe vaccine-enhanced RSV disease including weight loss, eosinophilia, and lung histopathology while it enhanced protection against RSV over unadjuvanted FI-RSV (Kim KH, et al. 2015).

[0048] Inactivated RSV adjuvanted with LPS, Poly(I:C), or PolyU induced protective immunity against live RSV challenge (Delgado MF, et al. 2009); however, all the adjuvants are chosen because of the components in RSV, i.e., F protein can activate TLR4 pathway like LPS; Poly(I:C) and PolyU function as viral genome.

[0049] Bovine RSV

[0050] Bovine RSV is a key player in the bovine respiratory disease complex, which is one of the most economically important and outstanding welfare issues in industrialized beef cattle production. The BRSV genome is very similar to the RSV genome (Hagglund S., et al. 2014).

[0051] When BRSV-immunostimulating complex (ISCOM) was used to immunize calves, IgG antibody specific for F, G, N, and SH are of high titers; it is to be noted that no significantly antibody responses against M and P were detected (Hagglund S., et al. 2014).

[0052] The present invention discovered a safe and effective RSV vaccine, where the vaccine comprises a recombinant fusion protein antigen. The recombinant fusion protein antigen comprises a phosphoprotein (P) moiety and a flagellin moiety, where the P moiety and flagellin moiety are covalently coupled so as to form a linear polypeptide. A “fusion protein” is a chimeric molecule in which the constituent moieties are all polypeptides and are attached (fused) to each other such that the chimeric molecule forms a continuous single chain. The various constituents can be directly attached to each other or can be coupled through one or more peptide linkers. A “linker” as used in reference to a chimeric molecule refers to any molecule that links or joins the constituent moieties of the chimeric molecule. Where the chimeric molecule is a fusion protein, the linker may be a peptide that joins the proteins comprising a fusion protein.

[0053] Referring now to FIG 1, the exemplary sequences of P protein include RSV A2 strain (SEQ ID NO 1 for its nucleotide sequence and SEQ ID NO 2 for its amino acid sequence) and RSV mutant cpt-248 strain (SEQ ID NO 3 for its nucleotide sequence and SEQ ID NO 4 for its amino acid sequence). It is to be noted that P protein from RSV mutant cpt-248 strain shares 98-99% identity with that from other RSV A strains, and more than 90% identity with that from RSV B strains.

[0054] Referring now to FIG 2(a), there is provided a schematic diagram of the functional blocks in the recombinant fusion protein antigen in accordance with one embodiment of the present invention. The recombinant protein antigen **1** comprises a phosphoprotein (P) moiety **10** and a flagellin moiety **20**, where the P moiety **10** and flagellin moiety **20** are linearly covalent coupled. In certain embodiments, the flagellin moiety **20** uses its N-terminal to couple

with the C terminal of the P moiety **10** (as shown in FIG 2(a)). In certain embodiments, the flagellin moiety **20** uses its C-terminal to couple with the N-terminal of the P moiety **10** (switched positions in related to FIG 2(a)).

[0055] In certain embodiments, the P moiety **10** is a polypeptide represented by SEQ ID NO 2 or SEQ ID NO 4. In certain embodiments, the P moiety **10** is a polypeptide that shares at least 98%, preferably 99% identity to the polypeptide represented by SEQ ID NO 2 or SEQ ID NO 4.

[0056] In certain embodiments, the flagellin moiety **20** is a polypeptide represented by SEQ ID NO 8. In certain embodiments, the flagellin moiety **20** is a polypeptide that shares at least 98%, preferably 99% identity to the polypeptide represented by SEQ ID NO 8.

[0057] In certain embodiments, the recombinant fusion protein antigen **1** further comprises a first linker **30** coupling the P moiety **10** and flagellin moiety **20**, where the first linker **30** is an amino acid or a peptide of two or more amino acids; the amino acid(s) in the first linker **30** shall be flexible without large side residues to avoid immune responses. In addition, the first linker **30** shall be resistant to enzymatic digestion so as to stabilize the recombinant fusion protein antigen when such a vaccine is administered.

[0058] In certain embodiments, the recombinant fusion protein antigen **1** further comprises a purification tag **40** for facilitating the purification of the recombinant fusion protein antigen **1**. The purification tag **40** can be disposed at either the N-terminal or C-terminal of the recombinant fusion protein antigen **1**. For example, six histidine residues are fusedly located at its N- or C-terminals, allowing purification using Ni²⁺ column. After the purification, the six histidine residues can be removed by chemical or enzymatic cleavage. In fact, any known purification tag is suitable here including myc tag, HA tag, Flag-peptide, KT3 epitope, alpha-tubulin epitope, T7 gene 10 protein peptide tag, glutathione-S-transferase (GST), strep-tag, bovine pancreatic trypsin inhibitor (BPTI), and maltose binding protein (MBP).

[0059] In certain embodiments, the recombinant fusion protein antigen **1** further comprises a second linker **50** linearly coupling the purification tag **40** with the P moiety **10** or flagellin moiety **20**. In certain embodiments, the second linker **50** is a cleavable linker that is composed of an amino acid or a sequence of amino acids, which can be cleaved chemically or enzymatically at its C-terminal. In the expression vectors, the cleavable linker comprises a DNA sequence which codes for an amino acid or a sequence of amino acids which can be cleaved chemically or enzymatically at its C-terminal.

[0060] Examples of chemical agents useful for cleaving proteins are cyanogen bromide, 2-(2-nitrophenylsulfenyl)-3-bromo-3'-methylindolinium (BNPS-skatole), hydroxylamine, and the like. Cyanogen bromide cleaves proteins at the C-terminal of a methionine residue. BNPS-skatole cleaves at the C-terminal of a tryptophan residue. Hydroxylamine cleaves at the C-terminal of the moiety –Asn-Z- in which Z is Gly, Leu, or Ala.

[0061] Examples of enzymatic agents useful for cleavage are trypsin, papain, pepsin, plasmin, thrombin, enterokinase, and the like. Each effects cleavage at a particular amino acid sequence which it recognizes. Enterokinase, for example, recognizes the amino acid sequence – (Asp)_n-Lys- in which n is an integer from 2 to 4.

[0062] As discussed above, the techniques for expression vector cloning, construction and amplification are well known to those skilled in the art. Therefore, the expression vectors for the recombinant fusion protein antigen can be constructed by routine procedures; no further details are provided herein in order not to obscure the principles of the present invention.

[0063] The RSV vaccine of the present invention further comprises a pharmaceutically acceptable solution including such as saline, PBS. In certain embodiments, the RSV vaccine may include other components such as other proteins from RSV.

[0064] Examples

[0065] 1. Preparation of virus for challenge

[0066] RSV stain A2 was propagated in Hep-2 cells; after sonication, centrifuged at 1000g, 4°C for 10min; collected supernatants; ultra-centrifuged at 80,000g, 4°C for 2hs to purify viruses. The purified viruses were titrated by plaque assay, and stored for challenges.

[0067] 2. Preparation of FI-RSV

[0068] Added neutral formalin into purified RSV A2 virus suspension solution at 1:4,000, and incubated at 37°C for 3 days with rotation; then centrifuged at 30,000rpm for 30 min; the pellets were resuspended into a volume of 1/25 of the original volume; then mixed FI-RSV with adjuvant Alum (4mg/ml) and incubated at room temperature for 24 hours.

[0069] 3. Preparation of recombinant proteins

[0070] Referring now to FIG 1, there are provided the sequences of recombinant P protein with His tag (nucleotide sequence of SEQ ID NO 5 and amino acid sequence of SEQ ID NO 6), recombinant flagellin KFD1 (nucleotide sequence of SEQ ID NO 7 and amino acid sequence of SEQ ID NO 8), and recombinant P-KFD1 (nucleotide sequence of SEQ ID NO 9 and amino acid sequence of SEQ ID NO 10). The DNA fragments were cloned into pET28a vector using primers (SEQ ID NOS 11-16), and the resultant pET28a-P, pET28a-KFD1, and

pET28a-P-KFD1 expression plasmids were transformed into bacteria following conventional protocols. All expression recombinant proteins were verified by western blot (FIG 2(b)) and purified using their His tag. Endotoxins were removed before use.

[0071] Immunization

[0072] Referring now to FIG 3, there are provided the temporal protocols of immunization and manipulations. Groups of BALB/c mice (n=18) were immunized three times at days 0, 25 or 50: (a) 10µl saline (i.n); (c) 10µl P protein (40µg) and 2µl CTB adjuvant (2µg, Sigma) mixture (i.n); (d) 12µl P-KFD1 protein (24µg) (i.n). For group (b), they were first immunized at day 42 and boosted two weeks later, 100µl FI-RSV vaccine containing 2µg FI-RSV and 400µg alum adjuvant (i.m). Sera were collected at days 21, 39 or 64; the titers of neutralizing antibodies were assayed.

[0073] Challenge

[0074] Challenge was done at day 25 after the last immunization. All mice were anesthetized by Avertin (tribromoethanol dissolved in tert-pentyl alcohol), and i.n. infected with 1×10^7 PFU RSV A2 in a volume of 50µl. The antigens and conditions for immunization and challenges are summarized in Table 1.

[0075] Table 1. Summary of antigens and conditions for immunization and challenges

group	Number of mice	Route/dose	challenge doses	challenge volume
(a) Saline	18	i.n./saline-diluted anesthetic	1×10^7 PFU	50 µl
(b) FI-RSV	18	i.m. at Day 42 (2x)/2µg FI-RSV +400µg Alum	1×10^7 PFU	50 µl
(c) P+CTB	18	i.n.(3x)/ 40µg P + 2µg CTB	1×10^7 PFU	50 µl
(d) P-KFD1	18	i.n.(3x)/ 24µg P-KFD1	1×10^7 PFU	50 µl

[0076] Immune plaque assay for determining virus titers in nasal and lung tissues

[0077] At day 4 post challenge, mice were sacrificed, and nose and lung tissues were collected, and homogenized using a homogenizer. In 24-well plates, Vero cells were inoculated, and grew into 100% confluence; then diluted homogenized samples were added into the wells, and incubated at 37°C for 1 hour; after washing 3 times, added into wells 1ml DMEM culture media containing 2% FBS, 1% P+S, 0.75% methyl cellulose; then incubated at an incubator for 4-5 days. Then, gently discarded culture media from the wells; fixed the wells with

paraformaldehyde and Triton X-100 for 20 minutes; added P protein-specific monoclonal antibody; incubated for 2 hours; added Goat anti-mouse IgG-HRP; incubated for 1 hour; and finally developed with AEC substrate, and counted the plaques.

[0078] Titration of serum neutralization antibody titers

[0079] Sera were inactivated; then diluted sequentially at 2-fold; the diluted serum samples (100 μ l) were incubated with 200 PFU of RSV A2 virus (100 μ l) at 37°C for 1 hour; then added onto Vero cell monolayer; allowed to adhere for 1 hour; incubated for 3 days; determined virus titers using immune plaque assay as described above. Serum neutralization antibody titer is defined as the serum inverse dilution folds that can cause a 50% reduction of plaque numbers, i.e. 50% plaque reduction neutralization titer (PRNT).

[0080] Measurement of airway responsiveness in mice

[0081] Day 4 post-challenge, anesthetized the mice with pentobarbital sodium, cut open the neck skins of the mice with surgical scissors, stripped the trachea, inserted tracheal intubation into the stripped trachea, connected the other end of tracheal intubation to a ventilator, and set the respiration rate at 1.5:1, and ventilator passive respiratory frequency for 90 times/minute.

[0082] After that, the mice were quickly transferred to the volume scan box, and the trachea was connected with the airway of the volume scan box. Starting AniRes2005 operating software to observe the airway pressure change curve and adjusting air quantity to mice to the level at which mouse's spontaneous breathing could not resist the effects of ventilator passive respiratory. After the pressure change curve became smooth and regular, the skin and muscle of the neck side of the mouse were separated, and the vein puncture was carried out to fix the needle so as to establish an intravenous drug administration passage. After intravenous injection of methacholine (MCH) excitation at sequential concentrations of 0.025, 0.05, 0.1, 0.2mgMCH/kg, used the analysis software to simultaneously record mouse airway inspiratory resistance (RI), expiratory resistance (RE) and pulmonary adaptation (cdyn) change curves.

[0083] Digestion of lung tissue

[0084] The mice were anesthetized with pentobarbital sodium, and the heart was perfused with 5ml PBS from the right atrium. The lung tissue was separated and cut to 1-2 mm². The lung tissue was digested by 2ml digestion buffer at 37°C for 40 min. The formula of the digestion buffer: HBSS culture medium containing Collagenase I (125 U/ml) and DNase I (30 U/ml). After the digestion, the lung tissue was gently rubbed and filtered to obtain single cell suspension, and then separated by Percoll separation solution, so as to get the lymphocytes and mononuclear cells.

[0085] Flow cytometry method

[0086] For detecting various types of cell infiltration in the lung, cells were surface stained with the following specific monoclonal antibodies: FITC Anti-Mouse F4/80, PE-Cy7 Anti-Mouse Gr-1, APC-EF780 Anti-Mouse CD11b, APC Anti-Mouse CD45, PE Anti-Mouse Siglec F; the staining was at 4°C for 30min, and then the cells were fixed.

[0087] Analysis of T cell response using ELISPOT assay

[0088] At day 4 post challenge, the lung and spleen were taken, and lymphocytes were separated. Pre-coated IFN- γ ELISPOT plates after activation, were inoculated lung lymphocytes and spleen lymphocytes, and stimulated by P protein. The positive and negative controls were set up. Then the plates were incubated in the incubator for 48 hours, subsequently incubated with secondary antibody with AEC substrate color, with a multifunctional cell analysis and enzyme linked spot analysis system to compute plaques.

[0089] Histology

[0090] Lungs were fixed with formalin. After fixation, tissue sections were prepared following standard procedures. Some slides were stained with hematoxylin-eosin (H&E) to identify cellular infiltrates.

[0091] Results

[0092] Referring now to FIG 4, there are provided graphs showing the P-specific IgG and IgA antibody titers in serum and vaginal samples from the groups of immunization with P+CTB or P-KFD1. It is apparent that the group of immunization with P+CTB showed higher titers than the group of P-KFD1.

[0093] Referring now to FIG 5, there are provided graphs showing viral loads in (a) nose or (b) lung after challenge.

[0094] Referring now to FIG 6, there is provided a graph showing the P-specific T cell responses. The group of P+CTB immunization showed significant T cell response; in contrast, the group of P-KFD1 immunization showed insignificant T cell response.

[0095] Referring now to FIG 7, there is provided a panel of pictures showing the lung tissues: (a) normal and uninfected; (b) control group immunized with saline and infected with RSV; (c) FI-RSV immunized group infected with RSV; (d) P+CTB immunized group infected with RSV; and (e) P-KFD1 immunized group infected with RSV. All sections from immunized groups were from mice at day 7 post infection. The saline and FI-RSV groups showed a large number of interstitial pneumonia and bronchitis; the P+CTB group had a large number of

lymphocytic infiltrations; and surprisingly the P-KFD1 group had no apparent pathological lesions.

[0096] Referring now to FIG 8, there is provided a graph showing the weight changes after challenge. The P-KFD1 group showed evident less weight loss than the other groups.

[0097] Referring now to FIG 9, there are provided graphs: (a) showing the correlation of inspiratory resistance (RI) with different concentrations of methacholine (MCH); and (b) showing the correlation of expiratory resistance (RE) with different concentrations of methacholine (MCH) in different groups after challenge. The P-KFD1 group showed better RI and RE values than the FI-RSV and P+CTB groups.

[0098] Referring now to FIG 10, there are provided graphs showing lymphocyte infiltration into lung tissues at day 8 post challenge: (a) immunocyte numbers; (b) neutrophil frequency in lung; (c) eosinophil frequency in lung; and (d) recruited macrophage frequency in lung. P-KFD1 group has less immunocyte infiltration than P+CTB group. P+KFD1 group showed no significant infiltrated eosinophils and macrophages.

[0099] While the present invention has been described with reference to particular embodiments, it will be understood that the embodiments are illustrative and that the invention scope is not so limited. Alternative embodiments of the present invention will become apparent to those having ordinary skill in the art to which the present invention pertains. Such alternate embodiments are considered to be encompassed within the scope of the present invention. Accordingly, the scope of the present invention is defined by the appended claims and is supported by the foregoing description.

References

Cullen LM, Blanco JC, Morrison TG. Cotton rat immune responses to virus-like particles containing the pre-fusion form of respiratory syncytial virus fusion protein. *J Transl Med.* 2015 Nov 5;13:350.

Delgado MF¹, Coviello S, Monsalvo AC, Melendi GA, Hernandez JZ, Batalle JP, Diaz L, Trento A, Chang HY, Mitzner W, Ravetch J, Melero JA, Irusta PM, Polack FP. Lack of antibody affinity maturation due to poor Toll-like receptor stimulation leads to enhanced respiratory syncytial virus disease. *Nat Med.* 2009 Jan;15(1):34-41.

Green CA, Scarselli E, Sande CJ, Thompson AJ, de Lara CM, Taylor KS, Haworth K, Del Sorbo M, Angus B, Siani L, Di Marco S, Traboni C, Folgori A, Colloca S, Capone S, Vitelli A, Cortese R, Klenerman P, Nicosia A, Pollard AJ. Chimpanzee adenovirus- and MVA-vectored

respiratory syncytial virus vaccine is safe and immunogenic in adults. *Sci Transl Med*. 7:300ra126 (2015)

Groothuis JR, King SJ, Hogerman DA, Paradiso PR, Simoes EA. Safety and immunogenicity of a purified f protein respiratory syncytial virus (pfp-2) vaccine in seropositive children with bronchopulmonary dysplasia. *The Journal of infectious diseases*. 1998; 177(2):467–469.

Hägglund S, Hu K, Blodörn K, Makabi-Panzu B, Gaillard AL, Ellencrona K, Chevret D, Hellman L, Bengtsson KL, Riffault S, Taylor G, Valarcher JF, Eléouët JF. Characterization of an experimental vaccine for bovine respiratory syncytial virus. *Clin Vaccine Immunol*. 21: 997-1004 (2014).

Kim K.H., Lee Y.T., Hwang H.S., Kwon Y.M., Jung Y.J., Lee Y., Lee J.S., Lee Y.N., Park S., Kang S.M. Alum adjuvant enhances protection against respiratory syncytial virus but exacerbates pulmonary inflammation by modulating multiple innate and adaptive immune cells. *PLOS ONE*. 10: e0139916 (2015).

Lee S, Quan FS, Kwon Y, Sakamoto K, Kang SM, Compans RW, Moore ML. Additive protection induced by mixed virus-like particles presenting respiratory syncytial virus fusion or attachment glycoproteins. *Antiviral Res*. 2014 Nov;111:129-35.

Schepens B¹, Sedeyn K², Vande Ginste L², De Baets S², Schotsaert M², Roose K², Houspie L³, Van Ranst M³, Gilbert B⁴, van Rooijen N⁵, Fiers W², Piedra P⁶, Saelens X¹. Protection and mechanism of action of a novel human respiratory syncytial virus vaccine candidate based on the extracellular domain of small hydrophobic protein. *EMBO Mol Med*. 2014 Oct 8;6(11):1436-54.

Yang K. and S.M. Varga. Mucosal vaccines against respiratory syncytial virus. *Curr Opin Virol*. 6:78-84 (2014).

RESPIRATORY SYNCYTIAL VIRUS VACCINE

Abstract

A respiratory syncytial virus (RSV) vaccine comprising a recombinant fusion protein antigen. In one embodiment, the recombinant fusion protein antigen comprises a phosphoprotein (P) moiety, wherein the P moiety is a polypeptide that shares at least 98% identity to the polypeptide represented by SEQ ID NO 2 or SEQ ID NO 4; and a flagellin moiety, wherein the flagellin moiety is a polypeptide that shares at least 98% identity to the polypeptide represented by SEQ ID NO 8; whereby the P moiety and flagellin moiety are covalently coupled so as to form a linear polypeptide.

CLAIMS

What is claimed is:

1. A respiratory syncytial virus (RSV) vaccine comprising a recombinant fusion protein antigen; wherein the recombinant fusion protein antigen comprises:
 - a phosphoprotein (P) moiety, wherein the P moiety is a polypeptide that shares at least 98% identity to the polypeptide represented by SEQ ID NO 2 or SEQ ID NO 4; and
 - a flagellin moiety, wherein the flagellin moiety is a polypeptide that shares at least 98% identity to the polypeptide represented by SEQ ID NO 8;where the P moiety and flagellin moiety are covalently coupled so as to form a linear polypeptide.
2. The RSV vaccine of claim 1, wherein the P moiety is a polypeptide that shares at least 99% identity to the polypeptide represented by SEQ ID NO 2 or SEQ ID NO 4; and wherein the flagellin moiety is a polypeptide that shares at least 99% identity to the polypeptide represented by SEQ ID NO 8.
3. The RSV vaccine of claim 1, wherein the P moiety is a polypeptide represented by SEQ ID NO 2 or SEQ ID NO 4; and wherein the flagellin moiety is a polypeptide represented by SEQ ID NO 8.
4. The RSV vaccine of claim 1, wherein the recombinant fusion protein antigen further comprises a first linker coupling the P moiety and flagellin moiety; wherein the first linker is an amino acid or a peptide of two to fifteen amino acids.
5. The RSV vaccine of claim 1, wherein the recombinant fusion protein antigen further comprises a purification tag for facilitating the purification of the recombinant fusion protein antigen, wherein the purification tag is disposed at either the N-terminal or C-terminal of the recombinant fusion protein antigen.
6. The RSV vaccine of claim 5, wherein the purification tag is composed of six histidine residues.

7. The RSV vaccine of claim 5, wherein the recombinant protein antigen further comprises a second linker linearly coupling the purification tag with the P moiety or flagellin moiety.
8. The RSV vaccine of claim 7, wherein the second linker is a cleavable linker that is composed of an amino acid or a sequence of amino acids which can be cleaved chemically or enzymatically at its C-terminal.
9. The RSV vaccine of claim 1, wherein the recombinant fusion protein antigen is represented by SEQ ID NO 10.