IMMUNOGENIC MIDDLE EAST RESPIRATORY SYNDROME CORONAVIRUS (MERS-CoV) COMPOSITIONS AND METHODS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of US Provisional Application No. 61/880,111, filed September 19, 2013, which is herein incorporated by reference in its entirety for all purposes.

DESCRIPTION OF THE TEXT FILE SUBMITTED ELECTRONICALLY

[0002] The contents of the text file submitted electronically herewith are incorporated herein by reference in their entirety: A computer readable format copy of the Sequence Listing (filename: NOVV-056/01WO_Seqlist.txt, date recorded: September 18, 2014, file size 17,384 megabytes).

BACKGROUND

[0003] Coronaviruses can cause disease in animals such as humans. SARS (Severe Acute Respiratory Syndrome) is one example of such a disease. Recently, a new coronavirus has emerged, Middle East Respiratory Syndrome Coronavirus (MERS-CoV), which is associated with human fatalities. MERS-CoV has been identified as a "threat to global health" by the World Health Organization (WHO), and has been reported in 21 countries in the Middle East and Europe. MERS-CoV is a highly pathogenic respiratory virus that causes severe respiratory distress and potentially renal failure in infected individuals [1, 2].

[0004] Coronaviruses attach to the membrane of cells via interaction of their Spike (S) glycoproteins on the surface of the virion with their cognate receptor on host cells (e.g. dipeptidyl peptidase 4 (DPP4), found on a variety of human cells including lung and kidney cells). The S glycoprotein consists of an N-terminal S1 domain that contains the receptor binding domain (RBD) and an S2 domain responsible for virus-cell fusion. The MERS-CoV RBD consists of a core domain that has been co-crystallized with the human DPP4 protein, showing that it interacts with blades 4 and 5 of DPP4 [11]. In other coronaviruses, including SARS-CoV, antibodies to the RBD are able to neutralize and inhibit growth of the virus *in vitro* [12-14]. In mouse models of SARS-CoV, vaccine induced and passively transferred neutralizing antibodies have proven to be effective in inhibiting lung pathogenesis and death

[15]. However, Antibody Dependent Enhancement (ADE) in human immune cells has been reported for SARS CoV *in vitro* though its clinical significance *in vivo* is unknown.

[0005] Human convalescent plasma and/or immunoglobulin has been used effectively to prevent and treat many viral infectious agents [16]. Public Health England and the International Severe Acute Respiratory & Emerging Infection Consortium (ISARIC) identified passive immunotherapy with neutralizing antibodies (including convalescent plasma) as a MERS-CoV treatment approach that warrants priority study. However, production of large quantities of anti-pathogen human plasma and/or immunoglobulin with high affinity and avidity antibodies currently requires donations by convalescent humans that can limit widespread availability of these human derived products for a number of cultural, infrastructure, and logistical reasons. Alternative means to rapidly produce specific human polyclonal immunoglobulin to prevent and treat infectious agents, including MERS-CoV, are needed.

SUMMARY

[0006] Disclosed herein are compositions and methods for simulating immune responses against infection by the coronavirus (CoV) that causes Middle East Respiratory Syndrome (MERS-CoV). The present disclosure provides compositions that are immunogenic and provide protection against MERS-CoV infection. Advantageously, the compositions induce neutralizing antibodies against MERS-CoV and can be used as vaccines.

[0007] The present disclosure also provides for a method of inducing substantial immunity to virus infection in an animal susceptible to MERS-CoV, comprising administering at least one effective dose of the vaccine comprising a MERS-CoV nanoparticle. In one embodiment, said method comprises administering to an animal said nanoparticle orally, intradermally, intranasally, intramuscularly, intraperitoneally, intravenously, or subcutaneously.

[0008] In aspects, the nanoparticle contains only one type of MERS-CoV protein. In particular aspects, the sole protein is a Spike (S) protein.

[0009] In one aspect, the disclosure provides an immunogenic composition comprising a MERS-CoV nanoparticle, wherein the nanoparticle comprises at least one trimer of a Spike polypeptide. In another embodiment, the nanoparticle comprises at least about five trimers to about 30 trimers of the Spike polypeptide. In another embodiment, the nanoparticle

comprises at least about ten trimers to about 20 trimers of the Spike polypeptide. In one embodiment, the concentration of the nanoparticle is at least about 20 μg/mL to about 60 μg/mL. In another embodiment, the concentration of the nanoparticle is at least about 30 μg/mL to about 50 μg/mL. In a further embodiment, the Spike polypeptide comprises SEQ ID NO: 2. In yet a further embodiment, the Spike polypeptide consists of SEQ ID NO: 2.

[0010] In another aspect, the disclosure provides a method of producing a high affinity antibody comprising administering to an animal an immunogenic composition comprising a MERS-CoV nanoparticle, wherein the nanoparticle comprises at least one trimer of a Spike polypeptide, collecting serum and/or plasma from the animal, and purifying the antibody from the serum and/or plasma. In one embodiment, the method further comprises the administration of an adjuvant. In a further embodiment, the adjuvant is selected from the group consisting of Montanide ISA 206, Quil A, Alum, Freund's adjuvant, incomplete Freund's adjuvant, and aluminum hydroxide adjuvants. In one embodiment, the animal is bovine or equine. In a further embodiment, the bovine or equine animal is a transgenic animal.

[0011] In one embodiment, the antibody produced by immunization of an animal with the nanoparticle has a KD between 10^{-8} and 10^{-15}. In a further embodiment, the antibody has a KD between about 10^{-12} and 10^{-14} or between about 10^{-13} and 10^{-14}.

[0012] In yet another aspect, the disclosure comprises further administering the purified high affinity antibody produced by the method to a human subject.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] FIG. 1 illustrates the Coronavirus structure.

[0014] FIG. 2 illustrates vaccine particles obtained by performing methods disclosed herein.

[0015] FIG. 3 illustrates binding of antibody to MERS CoV nanoparticle VLPs and SARS CoV nanoparticle VLPs disclosed herein by anti-MERS antibody and by anti SARS antibody respectively.

[0016] FIG. 4 illustrates electron micrographs of MERS CoV nanoparticle VLPs and SARS CoV nanoparticle VLPs disclosed herein.

[0017] FIG. 5 illustrates an immunization protocol for MERS CoV nanoparticle VLPs and SARS CoV nanoparticle VLPs disclosed herein at different time periods, different doses and with and without adjuvant.

[0018] FIG.6 illustrates vaccine particles obtained by performing methods disclosed herein. The MERS Spike proteins are shown assembled in trimer of 26nm protein-protein micelles.

[0019] FIG.7 illustrates neutralization results following immunization of MERS CoV VLPs and SARS CoV nanoparticle VLPs disclosed herein into mice. The Matrix M adjuvant is especially effective.

[0020] FIG.8 illustrates a codon-optimized MERS CoV Spike nucleotide sequence.

[0021] FIG.9 illustrates a MERS CoV Spike amino acid sequence.

[0022] FIG. 10A-C shows a MERS spike protein specific antibody production and ELISA titers. Panel A shows the Vaccination strategy (v notes the vaccination number; e.g. v1 is the first vaccination, v2 is the second vaccination, etc). Panel B shows the antibody titers against recombinant MERS-CoV Spike protein in the serum samples from individual Tc animals over immunization period as measured by ELISA. The titer value (units/ml) is defined as the highest dilution of serum sample where the OD450 reading was 2.5-fold higher than blank. Panel C shows the antibody titers against recombinant MRS-CoV Spike protein in SAB-300 and SAB-301. The titer activity value (units/mg) is defined as the highest dilution of 1 mg of antibody where the OD450 reading was 2.5-fold higher than blank.

[0023] FIG. 11A-B shows the results from a Fluorescence reduction neutralization assay. Panel A shows vaccinated bovine sera was tested for neutralization by $FRNT_{50}$ assay. Mass (μg) of serum needed for 50% inhibition of MERS-CoV detected is graphed for each sample. Infection was quantified after pre-treatment of MERS-CoV with a dilution of sera. After infection, cells were fixed and labeled with anti-spike antibody to quantify the level at which only 50% of the cells were infected compared to mock sera. Panel B shows the $FRNT_{50}$ assay results for SAB-300 and SAB-301 which were assayed for their ability to inhibit infection of MERS-CoV in Vero cells. Antigen affinity purified rabbit Anti-Spike antibody was used as the positive control in these $FRNT_{50}$ assays. Stats: unpaired T-test with Welch's correction, assumed Gaussian distribution. No significance found. Bars are 95% CI

[0024] FIG. 12A-B shows MERS-CoV neutralization assays. Panel A shows SAB-300 and SAB-301 were tested for their ability to neutralize MERS-CoV by $TCID_{50}$ assay graphed

as percent of virus titer compared to negative control purified hIgG. A higher percentage means less inhibition of infection. Panel B shows antibody dependent enhancement of SAB-300 and SAB-301 on MERS-CoV. RNA from MERS-CoV pre-incubated with SAB-300 and SAB-301 at 48 hours post infection was assayed for amount of MERS-CoV specific transcript, and graphed as fold change compared to mock samples. n.s. means not-significant.

[0025] FIG. 13A-B shows inhibition of MERS-CoV replication *in vivo*. SAB-300 (A) or SAB-301 (B) was transferred into Ad5-hDPP4 transduced BALB/c mice (6wks, female) intraperitoneally 12 hours before MERS-CoV infection. Mice were then infected with 1×10^5 PFU MERS-CoV intranasally. Virus titers in the lungs were measured at the indicated time points. Titers are expressed as PFU/g tissue. n= 3 mice/group/time point. *P <0.05 compared to No Treatment group; P <0.05 compared to Control Ab group.

[0026] FIG. 14 shows the binding kinetics of MERS-CoV S protein to bovine Tc derived human IgG.

DETAILED DESCRIPTION

[0027] It should be understood that singular forms such as "a," "an," and "the" are used throughout this application for convenience, however, except where context or an explicit statement indicates otherwise, the singular forms are intended to include the plural. Further, it should be understood that every journal article, patent, patent application, publication, and the like that is mentioned herein is hereby incorporated by reference in its entirety and for all purposes. All numerical ranges should be understood to include each and every numerical point within the numerical range, and should be interpreted as reciting each and every numerical point individually. The endpoints of all ranges directed to the same component or property are inclusive, and intended to be independently combinable.

[0028] "About" includes all values having substantially the same effect, or providing substantially the same result, as the reference value. Thus, the range encompassed by the term "about" will vary depending on context in which the term is used, for instance the parameter that the reference value is associated with. Thus, depending on context, "about" can mean, for example, ±15%, ±10%, ±5%, ±4%, ±3%, ±2%, ±1%, or ± less than 1%. Importantly, all recitations of a reference value preceded by the term "about" are intended to also be a recitation of the reference value alone.

[0029] As used herein, the term "baculovirus," also known as baculoviridae, refers to a family of enveloped DNA viruses of arthropods, members of which may be used as expression vectors for producing recombinant proteins in insert cell cultures. The virion contains one or more rod-shaped nucleocapsids containing a molecule of circular supercoiled double-stranded DNA (Mr 54×10^6-154×10^6). The virus used as a vector is generally *Autographa californica* nuclear polyhedrosis virus (NVP). Expression of introduced genes is under the control of the strong promoter that normally regulates expression of the polyhedron protein component of the large nuclear inclusion in which the viruses are embedded in the infected cells.

[0030] As used herein, the term "derived from" refers to the origin or source, and may include naturally occurring, recombinant, unpurified, or purified molecules.

[0031] As used herein, the term "macromolecular protein structure" refers to the construction or arrangement of one or more proteins.

[0032] As used herein, the term "vaccine" refers to a preparation of dead or weakened pathogens, or of derived antigenic determinants that is used to induce formation of antibodies or immunity against the pathogen. A vaccine is given to provide immunity to the disease, for example, MERS which is caused by MERS CoV viruses or SARS, which is caused by SARS CoV viruses. The present disclosure provides vaccine compositions that are immunogenic and provide protection. In addition, the term "vaccine" also refers to a suspension or solution of an immunogen (*e.g.* nanoparticle VLP) that is administered to a vertebrate to produce protective immunity, i.e., immunity that reduces the severity of disease associated with infection.

[0033] As used herein the term "substantial immunity" refers to an immune response in which when nanoparticles are administered to a vertebrate there is an induction of the immune system in said vertebrate which results in the prevention of infection, amelioration of infection or reduction of at least one symptom related to virus infection in said vertebrate.

[0034] As used herein the term "adjuvant" refers to a compound that, when used in combination with a specific immunogen (*e.g.* a nanoparticle VLP) in a formulation, augments or otherwise alters or modifies the resultant immune response. Modification of the immune response includes intensification or broadening the specificity of either or both antibody and cellular immune responses. Modification of the immune response can also mean decreasing or suppressing certain antigen-specific immune responses.

[0035] As used herein the term "immune stimulator" refers to a compound that enhances an immune response *via* the body's own chemical messengers (cytokines). These molecules comprise various cytokines, lymphokines and chemokines with immunostimulatory, immunopotentiating, and pro-inflammatory activities, such as interleukins (*e.g.*, IL-1, IL-2, IL-3, IL-4, IL-12, IL-13); growth factors (*e.g.*, granulocyte-macrophage (GM)-colony stimulating factor (CSF)); and other immunostimulatory molecules, such as macrophage inflammatory factor, Flt3 ligand, B7.1; B7.2, etc. The immune stimulator molecules can be administered in the same formulation as the nanoparticle VLPs, or can be administered separately. Either the protein or an expression vector encoding the protein can be administered to produce an immunostimulatory effect.

[0036] As used herein an "effective dose" generally refers to that amount of the nanoparticle sufficient to induce immunity, to prevent and/or ameliorate virus infection or to reduce at least one symptom of infection and/or to enhance the efficacy of another dose of a nanoparticle. An effective dose may refer to the amount of the nanoparticle sufficient to delay or minimize the onset of an infection. An effective dose may also refer to the amount of the nanoparticle that provides a therapeutic benefit in the treatment or management of infection. Further, an effective dose is the amount with respect to the nanoparticles alone, or in combination with other therapies, that provides a therapeutic benefit in the treatment or management of a viral infection. An effective dose may also be the amount sufficient to enhance a subject's (*e.g.*, a human's) own immune response against a subsequent exposure to virus. Levels of immunity can be monitored, *e.g.*, by measuring amounts of neutralizing secretory and/or serum antibodies, *e.g.*, by plaque neutralization, complement fixation, enzyme-linked immunosorbent, or microneutralization assay. In the case of a vaccine, an "effective dose" is one that prevents disease or reduces the severity of symptoms.

[0037] As used herein the term "substantially protective antibody response" refers to an immune response mediated by antibodies against a virus, which is exhibited by a vertebrate (*e.g.*, a human), that prevents or ameliorates infection or reduces at least one symptom thereof. Nanoparticles can stimulate the production of antibodies that, for example, neutralizing antibodies that block viruses from entering cells, blocks replication of said virus by binding to the virus, and/or protect host cells from infection and destruction.

[0038] As used herein the term "substantially protective cellular response" refers to an immune response that is mediated by T-lymphocytes and/or other white blood cells against

virus, exhibited by a vertebrate (*e.g.*, a human), that prevents or ameliorates infection or reduces at least one symptom thereof. One important aspect of cellular immunity involves an antigen-specific response by cytolytic T-cells ("CTL"s). CTLs have specificity for peptide antigens that are presented in association with proteins encoded by the major histocompatibility complex (MHC) and expressed on the surfaces of cells. CTLs help induce and promote the destruction of intracellular microbes, or the lysis of cells infected with such microbes. Another aspect of cellular immunity involves an antigen-specific response by helper T-cells. Helper T-cells act to help stimulate the function, and focus the activity of, nonspecific effector cells against cells displaying peptide antigens in association with MHC molecules on their surface. A "cellular immune response" also refers to the production of cytokines, chemokines and other such molecules produced by activated T-cells and/or other white blood cells, including those derived from CD4+ and CD8+ T-cells.

[0039] As used herein the term "substantial immunity in a population-wide basis" refers to immunity as a result of nanoparticles administered to individuals in a population. The immunity in said individual in said population results in the prevention, amelioration of infection, or reduction of at least one symptom related to virus infection in said individual, and prevents the spread of said virus to others in the population. The term population is defined as group of individuals (*e.g.* schoolchildren, elderly, healthy individuals etc.) and may comprise a geographic area (*e.g.* specific cities, schools, neighborhoods, workplace, country, state, etc.).

[0040] As use herein, the term "antigenic formulation" or "antigenic composition" refers to a preparation which, when administered to a vertebrate, especially a mammal, will induce an immune response.

[0041] As use herein, the term "vertebrate" or "subject" or "patient" refers to any member of the subphylum cordata, including, without limitation, humans and other primates, including non-human primates such as chimpanzees and other apes and monkey species. Farm animals such as cattle, sheep, pigs, goats and horses; domestic mammals such as dogs and cats; laboratory animals including rodents such as mice, rats and guinea pigs; birds, including domestic, wild and game birds such as chickens, turkeys and other gallinaceous birds, ducks, geese, and the like are also non-limiting examples . The terms "mammals" and "animals" are included in this definition. Both adult and newborn individuals are intended to be covered.

Nanoparticle VLPs

[0042] One of the goals in vaccine development is the production of vaccines that stimulate the immune system against a pathogen, but are not themselves infectious. This has lead away from the use of whole virions in vaccines toward more minimalist compositions. However, these minimalist compositions present their own obstacles, and in particular often exhibit a decreased immunogenicity requiring the use of adjuvants and systems that boost the immune response. Nanoparticles comprising native viral proteins offer a means of immunization that harnesses the viruses' natural ability to enter the cell and stimulate an immune response.

[0043] Nanoparticles, as disclosed herein, are a particular type of virus-like particle (VLP). The nanoparticles contain viral proteins but do not contain any viral genetic material, and thus are not infectious themselves. The nanoparticles are formed by self-assembly of recombinantly-produced viral proteins and stimulate especially useful immune responses. Without being bound by theory, it is though that the size, repetitive structure, and particulate nature contribute to the potent immune responses. Notably, the potent immune response may be obtained even in the absence of an adjuvant.

Nanoparticle Structure

[0044] The nanoparticles contain viral proteins, such as viral capsid or coat proteins, arranged in a polymeric form. Typically, the polymer is at least a trimer of viral proteins. In other aspects the polymer may contain more than three monomers of viral proteins. For example, the polymer may contain 4, 5, 6, 7, 8, 9 or 10 monomer units (referred to as a 4-mer, 5-mer, 6-mer and so on). In particular aspects, the polymers may be assembled into higher order structures. Thus, for example, the nanoparticles may contain at least about 3 polymers, at least about 5 polymers, at least about 10 polymers, at least about 15 polymers, at least about 20 polymers, or at least about 30 polymers. In particular aspects, the nanoparticle contains between about 5 and 15 polymers, between about 5 and 20 polymers, or between about 5 and 30 polymers. In yet other aspects, the nanoparticle contains between about 5 and 200 polymers, between about 10 and 200 polymers or between about 10 and 50 polymers. Thus, in particular examples, the nanoparticles may contain about 5 to about 20 trimers.

[0045] Generally, the more complex higher order structures may be obtained by increasing the protein concentration. In one example, lower protein concentrations yield nanoparticles comprising about 5 polymers, while higher protein concentrations (e.g. 30-50 μg/mL) yield nanoparticles comprising about 10 to 20 polymers. In one non-limiting example, the concentration of the nanoparticles is at least about 10 μg/mL, about 20 μg/mL, about 30 μg/mL, about 40 μg/mL, about 50 μg/mL, about 60 μg/mL, about 100μg/mL, about 200 μg/mL, or about 500 μg/mL. In particular, the concentration of the nanoparticles may be between about 10 μg/mL to about 1 mg/mL, or between about 20 μg/mL to about 500 μg/mL, or between about 30 μg/mL to about 100 μg/mL, or between about 30 μg/mL to about 50 μg/mL.

Nanoparticle Proteins

[0046] The nanoparticles disclosed herein can comprise any MERS-CoV protein, including, but not limited to the Spike (S) protein, the Membrane (M) protein, the Nucelocapsid (N) protein, and Envelope (E) protein, or a combination thereof. The proteins may be obtained or derived from any MERS-CoV strain, clade, or species. The nanoparticles can comprise one or more proteins from one or more MERS-CoV isolates, strains, clades, and/or species. In one embodiment, the proteins are obtained or derived from the Jordan-N3/2012 strain (GenBank KC776174.1) of MERS-CoV. In another embodiment, the proteins are obtained or derived from the Munich_2013 strain of MERS-CoV. In another embodiment, the proteins are obtained or derived from the Al-Hasa_1_2013 strain of MERS-CoV. The proteins comprising the nanoparticles may also be derived or obtained from a variety of other sources, including but not limited to, the Hafr-Al-batin_1_2013 strain, the Bisha_1_2012 strain, the Qatar_3_2013 strain, the Camel_Egypt_2013 strain, MERS-CoV clade A viruses, MERS-CoV clade B viruses, the closely related HKU4 and HKU5 bat coronavirus strains, and/ or other closely related coronaviruses. The MERS-CoV proteins may also be produced synthetically or recombinantly *in vitro*. In one embodiment, the proteins are produced recombinantly in insect cells, such as Sf9 cells.

[0047] In one embodiment, the polymer may comprise one or more different types of MERS-CoV proteins. In a further embodiment, the polymer comprises a single type of MERS-CoV protein. In a preferred embodiment, the polymer comprises Spike protein only. In another embodiment, the protein in a polymer consists of Spike protein. In a further

embodiment, the nanoparticle does not contain membrane or nucleocapsid proteins. In another further embodiment, the nanoparticle does not comprise any viral nucleic acid sequences.

[0048] In one embodiment, the nanoparticle comprises a Spike protein or a fragment thereof. In another further embodiment, the nanoparticle comprises a trimer of Spike proteins, or fragments thereof. In another embodiment, the nanoparticle comprises at least one polymer of Spike protein encoded by SEQ ID NO: 1. In another embodiment, the nanoparticle comprises at least one polymer of Spike protein comprising SEQ ID NO: 2. In another embodiment, the nanoparticle comprises at least one polymer of Spike protein consisting of SEQ ID NO: 2. In a further embodiment, the nanoparticle comprises at least one polymer of the receptor binding domain (RBD) of the Spike protein.

[0049] Nanoparticles may be prepared as described in the art (e.g. as described in US20100239617, published, Sep 23, 2010 and incorporated herein for all purposes). General texts which describe molecular biological techniques, which are applicable to the present disclosure, such as cloning, mutation, cell culture and the like, include Berger and Kimmel, Guide to Molecular Cloning Techniques, Methods in Enzymology volume 152 Academic Press, Inc., San Diego, Calif. (Berger); Sambrook *et al.*, Molecular Cloning--A Laboratory Manual (3rd Ed.), Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 2000 ("Sambrook") and Current Protocols in Molecular Biology, F. M. Ausubel *et al.*, eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., ("Ausubel"). These texts describe mutagenesis, the use of vectors, promoters and many other relevant topics related to cloning and mutation of proteins. Thus, the disclosure also encompasses using known methods of protein engineering and recombinant DNA technology to improve or alter the characteristics of the proteins expressed on or in the nanoparticles. They include but are not limited to site-directed, random point mutagenesis, homologous recombination (DNA shuffling), mutagenesis using uracil containing templates, oligonucleotide-directed mutagenesis, phosphorothioate-modified DNA mutagenesis, mutagenesis using gapped duplex DNA or the like. Additional suitable methods include point mismatch repair, mutagenesis using repair-deficient host strains, restriction-selection and restriction-purification, deletion mutagenesis, mutagenesis by total gene synthesis, double-strand break repair, and the like. Mutagenesis, *e.g.*, involving chimeric constructs, is also included in the present disclosure. In one embodiment,

mutagenesis can be guided by known information of the naturally occurring molecule or altered or mutated naturally occurring molecule, *e.g.*, sequence, sequence comparisons, physical properties, crystal structure or the like.

MERS Spike Proteins

[0050] Suitable Spike proteins or fragments thereof are obtained or derived from MERS-CoV isolates, strains, clades, and/or sequences. Alternatively, they can be produced recombinantly or synthetically. For example, a suitable MERS-CoV amino acid sequence is disclosed in Genbank Accession No. AGN70962 (Fig. 9 (SEQ ID NO: 2)). In one embodiment, the Spike protein or fragment thereof is obtained from an Al-Hasa MERS-CoV strain.

[0051] The disclosure provides variants of the MERS-CoV proteins. The variants may contain alterations in the amino acid sequences of the constituent proteins. The term "variant" with respect to a polypeptide refers to an amino acid sequence that is altered by one or more amino acids with respect to a reference sequence. The variant can have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties, *e.g.*, replacement of leucine with isoleucine. Alternatively, a variant can have "nonconservative" changes, *e.g.*, replacement of a glycine with a tryptophan. Analogous minor variations can also include amino acid deletion or insertion, or both. Guidance in determining which amino acid residues can be substituted, inserted, or deleted without eliminating biological or immunological activity can be found using computer programs well known in the art, for example, DNASTAR software.

[0052] Natural variants can occur due to antigenic drifts. Antigenic drifts are small changes in the viral proteins that happen continually over time. Thus, a person infected with a particular MERS-CoV virus strain develops antibodies against that virus, as newer virus strains appear, the antibodies against the older strains no longer recognize the newer virus and reinfection can occur. These naturally occurring MERS-CoV proteins and RBD variants may be used to produce nanoparticles as described herein.

[0053] In some embodiments, mutations containing alterations which produce silent substitutions, additions, or deletions, but do not alter the properties or activities of the encoded protein or how the proteins are made. Nucleotide variants can be produced for a variety of reasons, *e.g.*, to optimize codon expression for a particular host (change codons in

the human mRNA to those preferred by insect cells such as Sf9 cells). See U.S. patent publication 2005/0118191, herein incorporated by reference in its entirety for all purposes. In a particular aspect, the preferred protein is encoded by a codon-optimized nucleotide sequence such as shown in Figure 8 (SEQ ID NO: 1). The nucleic acid and polypeptides may be at least 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to the sequences shown in Figs. 8 and 9 (SEQ ID NOS: 1 and 2).

[0054] In addition, the nucleotides can be sequenced to ensure that the correct coding regions were cloned and do not contain any unwanted mutations. The nucleotides can be subcloned into an expression vector (*e.g.* baculovirus) for expression in any cell. The above is only one example of how the viral proteins can be cloned. A person with skill in the art understands that additional methods are available and are possible.

[0055] This disclosure also provides for constructs and methods that will increase the efficiency of nanoparticle production. For example, removing cleavage sites from proteins in order to increase protein expression. Other methods comprise the addition of leader sequences for more efficient transporting. For example, a heterologous signal sequence can be fused to a MERS protein. In one embodiment, the signal sequence can be derived from the gene of an insect cell. In another embodiment, the signal peptide is the chitinase signal sequence, which works efficiently in baculovirus expression systems. In other embodiment, interchanging leader sequences between proteins can provide better protein transport.

[0056] Suitable vectors encoding MERS-CoV proteins or fragments thereof may be used. The vector may be, for example, a phage, plasmid, viral, or retroviral vector. In one embodiment, the vector is a recombinant baculovirus vector. The constructs and/or vectors that encode genes should be operatively linked to an appropriate promoter, such as the AcMNPV polyhedrin promoter (or other baculovirus), phage lambda PL promoter, the *E. coli* lac, phoA and tac promoters, the SV40 early and late promoters, and promoters of retroviral LTRs are non-limiting examples. Other suitable promoters will be known to the skilled artisan depending on the host cell and/or the rate of expression desired. The expression constructs will further contain sites for transcription initiation, termination, and, in the transcribed region, a ribosome binding site for translation. The coding portion of the transcripts expressed by the constructs will preferably include a translation initiating codon at the beginning and a termination codon appropriately positioned at the end of the polypeptide to be translated.

[0057] The expression vectors will preferably include at least one selectable marker. Such markers include dihydrofolate reductase, G418 or neomycin resistance for eukaryotic cell culture and tetracycline, kanamycin or ampicillin resistance genes for culturing in *E. coli* and other bacteria. Among vectors preferred are virus vectors, such as baculovirus, poxvirus (*e.g.*, vaccinia virus, avipox virus, canarypox virus, fowlpox virus, raccoonpox virus, swinepox virus, etc.), adenovirus (*e.g.*, canine adenovirus), herpesvirus, and retrovirus. Bacterial vectors may also be used. Exemplary bacterial vectors include, pQE70, pQE60 and pQE-9, pBluescript vectors, Phagescript vectors, pNH8A, pNH16a, pNH18A, pNH46A, ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5. Among preferred eukaryotic vectors are pFastBac1 pWINEO, pSV2CAT, pOG44, pXT1 and pSG, pSVK3, pBPV, pMSG, and pSVL. Other suitable vectors will be readily apparent to the skilled artisan.

[0058] Vectors, *e.g.*, vectors comprising MERS-CoV polynucleotides, can be transfected into host cells according to methods well known in the art. For example, introducing nucleic acids into eukaryotic cells can be by calcium phosphate co-precipitation, electroporation, microinjection, lipofection, and transfection employing polyamine transfection reagents. In one embodiment, the said vector is a recombinant baculovirus.

[0059] Recombinant vectors such as those disclosed above can be used to transfect, infect, or transform and can express proteins in eukaryotic cells and/or prokaryotic cells. Among eukaryotic host cells are yeast, insect, avian, plant, *C. elegans* (or nematode) and mammalian host cells. Non limiting examples of insect cells are, *Spodoptera frugiperda* (Sf) cells, *e.g.* Sf9, Sf21, *Trichoplusia ni* cells, *e.g.* High Five cells, and *Drosophila* S2 cells. Examples of fungi (including yeast) host cells are *S. cerevisiae, Kluyveromyces lactis* (*K. lactis*), species of Candida including *C. albicans* and *C. glabrata*, *Aspergillus nidulans*, *Schizosaccharomyces pombe* (S. pombe), *Pichia pastoris*, and *Yarrowia lipolytica*. Examples of mammalian cells are COS cells, baby hamster kidney cells, mouse L cells, LNCaP cells, Chinese hamster ovary (CHO) cells, human embryonic kidney (HEK) cells, and African green monkey cells, CV1 cells, HeLa cells, MDCK cells, Vero and Hep-2 cells. Xenopus laevis oocytes, or other cells of amphibian origin, may also be used. Prokaryotic host cells include bacterial cells, for example, *E. coli*, *B. subtilis*, and mycobacteria.

[0060] Methods to grow cells engineered to produce nanoparticles include, but are not limited to, batch, batch-fed, continuous and perfusion cell culture techniques. Cell culture means the growth and propagation of cells in a bioreactor (a fermentation chamber) where

cells propagate and express protein (*e.g.* recombinant proteins) for purification and isolation. Typically, cell culture is performed under sterile, controlled temperature and atmospheric conditions in a bioreactor. A bioreactor is a chamber used to culture cells in which environmental conditions such as temperature, atmosphere, agitation and/or pH can be monitored. In one embodiment, said bioreactor is a stainless steel chamber. In another embodiment, said bioreactor is a pre-sterilized plastic bag (*e.g.* Cellbag®, Wave Biotech, Bridgewater, NJ). In other embodiment, said pre-sterilized plastic bags are about 50 L to 1000 L bags.

[0061] The nanoparticles may then be isolated using methods that preserve the integrity thereof, such as by gradient centrifugation, *e.g.,* cesium chloride, sucrose and iodixanol, as well as standard purification techniques including, *e.g.*, ion exchange and gel filtration chromatography.

[0062] The following is an example of how nanoparticles can be made, isolated and purified. Usually nanoparticles are produced from recombinant cell lines engineered to create nanoparticles when said cells are grown in cell culture.

[0063] For example, production of nanoparticles can start by seeding Sf9 cells (non-infected) into shaker flasks, allowing the cells to expand and scaling up as the cells grow and multiply (for example from a 125-ml flask to a 50 L Wave bag). The medium used to grow the cell is formulated for the appropriate cell line (preferably serum free media, *e.g.* insect medium ExCell-420, JRH). Next, said cells are infected with recombinant baculovirus at the most efficient multiplicity of infection (*e.g.* from about 1 to about 3 plaque forming units per cell). Once infection has occurred, the proteins are expressed from the virus genome, self-assemble into nanoparticle VLPs and are secreted from the cells approximately 24 to 72 hours post infection. Usually, infection is most efficient when the cells are in mid-log phase of growth (4-8 $\times$ 10^6 cells/ml) and are at least about 90% viable.

[0064] Alternatively, nanoparticles may be produced, for example, by infecting host cells (e.g. Sf9) cells with a recombinant baculovirus comprising nucleotide sequences encoding the MERS-CoV protein or proteins or interest. The infected cells are incubated and harvested. The MERS-CoV protein (e.g. Spike protein) is extracted from the cellular membrane with a detergent. The proteins are then allowed to naturally assemble to form structures (e.g. trimers) which are then purified. The removal of the detergent during purification allows the proteins to form higher order structures such as nanoparticles comprising multiple structures

(e.g. protein-protein micellular nanoparticles comprising protein trimers). Generally, the number of structures in the nanoparticles is influenced by the protein concentration, with higher protein concentrations yielding nanoparticles comprising greater numbers of structures (e.g. trimers).

[0065] Nanoparticles can be harvested approximately 48 to 96 hours post infection, when the levels of nanoparticle VLPs in the cell culture medium are near the maximum but before extensive cell lysis. The Sf9 cell density and viability at the time of harvest can be about 0.5×10^6 cells/ml to about 1.5×10^6 cells/ml with at least 20% viability, as shown by dye exclusion assay. Next, the medium is removed and clarified. NaCl can be added to the medium to a concentration of about 0.4 to about 1.0 M, preferably to about 0.5 M, to avoid undesirable aggregation. The removal of cell and cellular debris from the cell culture medium containing nanoparticles can be accomplished by tangential flow filtration (TFF) with a single use, pre-sterilized hollow fiber 0.5 or 1.00 μm filter cartridge or a similar device.

[0066] Next, nanoparticles in the clarified culture medium can be concentrated by ultrafiltration using a disposable, pre-sterilized 500,000 molecular weight cut off hollow fiber cartridge. The concentrated nanoparticles can be diafiltrated against 10 volumes pH 7.0 to 8.0 phosphate-buffered saline (PBS) containing 0.5 M NaCl to remove residual medium components.

[0067] The concentrated, diafiltered nanoparticles can be furthered purified on a 20% to 60% discontinuous sucrose gradient in pH 7.2 PBS buffer with 0.5 M NaCl by centrifugation at 6,500 × g for 18 hours at about 4° C to about 10° C. Usually nanoparticle VLPs will form a distinctive visible band between about 30% to about 40% sucrose or at the interface (in a 20% and 60% step gradient) that can be collected from the gradient and stored. This product can be diluted to comprise 200 mM of NaCl in preparation for the next step in the purification process. This product contains nanoparticle VLPs and may contain intact baculovirus particles.

[0068] Further purification of nanoparticles can be achieved by anion exchange chromatography, or 44% isopycnic sucrose cushion centrifugation. In anion exchange chromatography, the sample from the sucrose gradient (see above) is loaded into column containing a medium with an anion (*e.g.* Matrix Fractogel EMD TMAE) and eluded via a salt gradient (from about 0.2 M to about 1.0 M of NaCl) that can separate the nanoparticle from

other contaminates (*e.g.* baculovirus and DNA/RNA). In the sucrose cushion method, the sample comprising the nanoparticles is added to a 44% sucrose cushion and centrifuged for about 18 hours at 30,000 g. Nanoparticles form a band at the top of 44% sucrose, while baculovirus precipitates at the bottom and other contaminating proteins stay in the 0% sucrose layer at the top. The nanoparticle peak or band is collected.

[0069] The intact baculovirus can be inactivated, if desired. Inactivation can be accomplished by chemical methods, for example, formalin or β-propyl lactone (BPL). Removal and/or inactivation of intact baculovirus can also be largely accomplished by using selective precipitation and chromatographic methods known in the art, as exemplified above. Methods of inactivation comprise incubating the sample containing the nanoparticles in 0.2% of BPL for 3 hours at about 25 °C to about 27 °C. The baculovirus can also be inactivated by incubating the sample containing the nanoparticles at 0.05% BPL at 4 °C for 3 days, then at 37 °C for one hour.

[0070] After the inactivation/removal step, the product comprising the nanoparticles can be run through another diafiltration step to remove any reagent from the inactivation step and/or any residual sucrose, and to place the nanoparticles into the desired buffer (*e.g.* PBS). The solution comprising nanoparticles can be sterilized by methods known in the art (*e.g.* sterile filtration) and stored in the refrigerator or freezer.

[0071] The above techniques can be practiced across a variety of scales. For example, T-flasks, shake-flasks, spinner bottles, up to industrial sized bioreactors. The bioreactors can comprise either a stainless steel tank or a pre-sterilized plastic bag (for example, the system sold by Wave Biotech, Bridgewater, NJ). A person with skill in the art will know what is most desirable for their purposes.

Pharmaceutical or Vaccine Formulations and Administration

[0072] The pharmaceutical compositions useful herein contain a pharmaceutically acceptable carrier, including any suitable diluent or excipient, which includes any pharmaceutical agent that does not itself induce the production of an immune response harmful to the vertebrate receiving the composition, and which may be administered without undue toxicity along with a nanoparticle. As used herein, the term "pharmaceutically acceptable" means being approved by a regulatory agency of the Federal or a state

government or listed in the U.S. Pharmacopia, European Pharmacopia or other generally recognized pharmacopia for use in vertebrates, and more particularly in humans.

[0073] Generally, the nanoparticles are administered in an effective amount or quantity (as defined above) sufficient to stimulate an immune response against one or more strains of MERS CoV virus. These compositions may be used as a vaccine and/or antigenic compositions for inducing a protective immune response in a vertebrate. The compositions may contain nanoparticles having different polymer types. For example, the compositions may contain primarily trimers, with the remainder being composed of different polymers. In one embodiment, the nanoparticles administered in an effective amount to stimulate an immune response against one or more strains of MERS-CoV virus comprise at least about 5 to 100 trimers, at least about 10 to 90 trimers, at least about 20 to 50 trimers, at least about 10 to 30 trimers, or at least about 10 to 20 trimers of MERS-CoV proteins. In a further composition embodiment, the nanoparticles administered comprise at least about 5 to 100 trimers, at least about 10 to 90 trimers, at least about 20 to 50 trimers, at least about 10 to 30 trimers, or at least about 10 to 20 trimers of Spike protein or a fragment thereof (such as RBD).

[0074] In one non-limiting embodiment, the concentration of the nanoparticles is at least about 10 μg/mL, about 20 μg/mL, about 30 μg/mL, about 40 μg/mL, about 50 μg/mL, about 60 μg/mL, about 100μg/mL, about 200 μg/mL, or about 500 μg/mL. In particular aspects, the concentration of the nanoparticles is between about 10 μg/mL to about 1 mg/mL, or between about 20 μg/mL to about 500 μg/mL, or between about 30 μg/mL to about 100 μg/mL, or between about 30 μg/mL to about 50 μg/mL.

[0075] In other examples, the compositions may contain higher order nanoparticles such as 5-mers to 6-mers. In particular aspects, the compositions contain at least 70% 5-mers to 6-mers, at least 80% 5-mers to 6-mers, or at least 90% 5-mers to 6-mers. In other aspects, the composition contains at least 70% 5-mers to 6-mers, at least 80% 5-mers to 6-mers, or at least 90% 5-mers to 6-mers.

[0076] In one embodiment, pharmaceutical formulations disclosed herein comprise nanoparticles comprising a MERS-CoV protein, often a Spike protein, and a pharmaceutically acceptable carrier or excipient.

[0077] In other embodiments, compositions may contain nanoparticles containing MERS-CoV proteins, or variants such as the RBD variant, from different MERS strains. Such

compositions may be administered to provide immunity to multiple, different strains. In an aspect, a composition may contain nanoparticles to provide an immune response against a first strain, second strain, a third strain. In another aspect, the nanoparticles may provide an immune response against four, five or six strains.In another embodiment, the pharmaceutical formulations comprise a purified, high affinity antibody produced in an animal administered with the nanoparticles.

[0078] Pharmaceutically acceptable carriers include but are not limited to saline, buffered saline, dextrose, water, glycerol, sterile isotonic aqueous buffer, and combinations thereof. A thorough discussion of pharmaceutically acceptable carriers, diluents, and other excipients is presented in Remington's Pharmaceutical Sciences (Mack Pub. Co. N.J. current edition). The formulation should suit the mode of administration. In a preferred embodiment, the formulation is suitable for administration to humans, preferably is sterile, non-particulate and/or non-pyrogenic.

[0079] The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. The composition can be a solid form, such as a lyophilized powder suitable for reconstitution, a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc.

[0080] The disclosure also provides for a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the immunogenic vaccine formulations. In a preferred embodiment, the kit comprises two containers, one containing nanoparticles and the other containing an adjuvant. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

[0081] Formulations may be packaged in a hermetically sealed container such as an ampoule or sachette indicating the quantity of composition. In one embodiment, the composition is supplied as a liquid, in another embodiment, as a dry sterilized lyophilized powder or water free concentrate in a hermetically sealed container and can be reconstituted, *e.g.*, with water or saline to the appropriate concentration for administration to a subject. Preferably, the composition is supplied as a dry sterile lyophilized powder in a hermetically

sealed container at a unit dosage of preferably, about 1 μg, about 5 μg, about 10 μg, about 20 μg, about 25 μg, about 30 μg, about 50 μg, about 100 μg, about 125 μg, about 150 μg, or about 200 μg. Alternatively, the unit dosage of the composition is less than about 1 μg, (for example about 0.08 μg, about 0.04 μg, about 0.2 μg, about 0.4 μg , about 0.8 μg , about 0.5 μg or less, about 0.25 μg or less, or about 0.1 μg or less), or more than about 125 μg, (for example about 150 μg or more, about 250 μg or more, or about 500 μg or more). These doses may be measured as total nanoparticles or as μg of MERS-CoV protein (e.g. Spike protein, or fragment thereof). The nanoparticle composition should be administered within about 12 hours, preferably within about 6 hours, within about 5 hours, within about 3 hours, or within about 1 hour after being reconstituted from the lyophylized powder.

[0082] In an alternative embodiment, a nanoparticle composition is supplied in liquid form in a hermetically sealed container indicating the quantity and concentration of the nanoparticle composition. Preferably, the liquid form of the nanoparticle composition is supplied in a hermetically sealed container at least about 50 μg/ml, more preferably at least about 100 μg/ml, at least about 200 μg/ml, at least 500 μg /ml, or at least 1 mg/ml.

[0083] The nanoparticles may be administered to an animal to elicit an immune response against MERS-CoV. In one embodiment, the animal is susceptible to MERS-CoV infection. In one embodiment, the animal is a human. Preferably, administration of the nanoparticles elicits substantial immunity against at least one MERS-CoV strain, isolate, clade, and/or species. In one embodiment, administration of the nanoparticles elicits substantial immunity against at least 2 or more MERS-CoV strains, isolates, clades, and/or species. In a further embodiment, administration of the nanoparticles elicits substantial immunity against another coronavirus. Typically, the dose can be adjusted within this range based on, *e.g.*, age, physical condition, body weight, sex, diet, time of administration, and other clinical factors.

[0084] Thus, disclosed herein are methods of formulating a vaccine or antigenic composition that induces substantial immunity to virus infection or at least one symptom thereof to a subject, comprising adding to said formulation an effective dose of a nanoparticle.

[0085] While stimulation of substantial immunity with a single dose is preferred, additional dosages can be administered, by the same or different route, to achieve the desired effect. In neonates and infants, for example, multiple administrations may be required to elicit sufficient levels of immunity. Administration can continue at intervals throughout childhood,

as necessary to maintain sufficient levels of protection against infection. Similarly, adults who are particularly susceptible to repeated or serious infection, such as, for example, health care workers, day care workers, family members of young children, the elderly, and individuals with compromised cardiopulmonary function may require multiple immunizations to establish and/or maintain protective immune responses. Levels of induced immunity can be monitored, for example, by measuring amounts of neutralizing secretory and serum antibodies, and dosages adjusted or vaccinations repeated as necessary to elicit and maintain desired levels of protection.

[0086] Thus, in one embodiment, a method to induce substantial immunity to virus infection or at least one symptom thereof in a subject, comprises administering at least one effective dose of a nanoparticle, wherein the nanoparticle comprises at least one trimer comprising MERS CoV Spike protein or fragment thereof. In other aspects the nanoparticle comprises at least one trimer consisting essentially of a MERS CoV Spike protein or a fragment thereof. Indeed, the Spike protein may be the only protein in the trimer and/or nanoparticle.

[0087] Methods of administering a composition comprising nanoparticles (vaccine and/or antigenic formulations) include, but are not limited to, parenteral administration (*e.g.*, intradermal, intramuscular, intravenous and subcutaneous), epidural, and mucosal (*e.g.*, intranasal and oral or pulmonary routes or by suppositories). In a specific embodiment, compositions of the present disclosure are administered intramuscularly, intravenously, subcutaneously, transdermally or intradermally. The compositions may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucous, colon, conjunctiva, nasopharynx, oropharynx, vagina, urethra, urinary bladder and intestinal mucosa, etc.) and may be administered together with other biologically active agents. In some embodiments, intranasal or other mucosal routes of administration of a composition comprising nanoparticles may induce an antibody or other immune response that is substantially higher than other routes of administration. In another embodiment, intranasal or other mucosal routes of administration of a composition comprising nanoparticles may induce an antibody or other immune response that will induce cross protection against other strains of viruses. Administration can be systemic or local. The prophylactic vaccine formulation is systemically administered, *e.g.*, by subcutaneous or intramuscular injection using a needle and syringe, or a needle-less

injection device. Alternatively, the vaccine formulation is administered intranasally, either by drops, large particle aerosol (greater than about 10 microns), or spray into the upper respiratory tract. While any of the above routes of delivery results in an immune response, intranasal administration confers the added benefit of eliciting mucosal immunity at the site of entry of the virus.

[0088] In yet another embodiment, the vaccine and/or antigenic formulation is administered in such a manner as to target mucosal tissues in order to elicit an immune response at the site of immunization. For example, mucosal tissues such as gut associated lymphoid tissue (GALT) can be targeted for immunization by using oral administration of compositions which contain adjuvants with particular mucosal targeting properties. Additional mucosal tissues can also be targeted, such as nasopharyngeal lymphoid tissue (NALT) and bronchial-associated lymphoid tissue (BALT).

[0089] Vaccines and/or antigenic formulations may also be administered on a dosage schedule, for example, an initial administration of the vaccine composition with subsequent booster administrations. In particular embodiments, a second dose of the composition is administered anywhere from two weeks to one year, preferably from about 1, about 2, about 3, about 4, about 5 to about 6 months, after the initial administration. Additionally, a third dose may be administered after the second dose and from about three months to about two years, or even longer, preferably about 4, about 5, or about 6 months, or about 7 months to about one year after the initial administration. The third dose may be optionally administered when no or low levels of specific immunoglobulins are detected in the serum and/or urine or mucosal secretions of the subject after the second dose. In a preferred embodiment, a second dose is administered about one month after the first administration and a third dose is administered about six months after the first administration. In another embodiment, the second dose is administered about six months after the first administration.

[0090] In another embodiment, a nanoparticle may be administered as part of a combination therapy. For example, nanoparticles may be formulated with other immunogenic compositions and/or antivirals.

[0091] The dosage of the pharmaceutical formulation can be determined readily by the skilled artisan, for example, by first identifying doses effective to elicit a prophylactic or therapeutic immune response, *e.g.*, by measuring the serum titer of virus specific

immunoglobulins or by measuring the inhibitory ratio of antibodies in serum samples, or urine samples, or mucosal secretions. Said dosages can be determined from animal studies.

[0092] In addition, human clinical studies can be performed to determine the preferred effective dose for humans by a skilled artisan. Such clinical studies are routine and well known in the art. The precise dose to be employed will also depend on the route of administration. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal test systems.

[0093] As also well known in the art, the immunogenicity of a particular composition can be enhanced by the use of non-specific stimulators of the immune response, known as adjuvants. Adjuvants have been used experimentally to promote a generalized increase in immunity against unknown antigens (*e.g.*, U.S. Pat. No. 4,877,611). Immunization protocols have used adjuvants to stimulate responses for many years, and as such, adjuvants are well known to one of ordinary skill in the art. Some adjuvants affect the way in which antigens are presented. For example, the immune response is increased when protein antigens are precipitated by alum. Emulsification of antigens also prolongs the duration of antigen presentation. Adjuvants may be included. Suitable adjuvants include those described in Vogel *et al.*, "A Compendium of Vaccine Adjuvants and Excipients (2nd Edition)," herein incorporated by reference in its entirety for all purposes.

[0094] Other exemplary adjuvants include complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed Mycobacterium tuberculosis), incomplete Freund's adjuvants and aluminum hydroxide adjuvant. Other adjuvants comprise GMCSP, BCG, aluminum hydroxide, MDP compounds, such as thur-MDP and nor-MDP, CGP (MTP-PE), lipid A, Montanide ISA 206, and monophosphoryl lipid A (MPL). RIBI, which contains three components extracted from bacteria, MPL, trehalose dimycolate (TDM) and cell wall skeleton (CWS) in a 2% squalene/Tween 80 emulsion also is contemplated. MF-59, Novasomes®, MHC antigens may also be used.

[0095] In an embodiment, the adjuvant is a paucilamellar lipid vesicle having about two to ten bilayers arranged in the form of substantially spherical shells separated by aqueous layers surrounding a large amorphous central cavity free of lipid bilayers. Paucilamellar lipid vesicles may act to stimulate the immune response several ways, as non-specific stimulators, as carriers for the antigen, as carriers of additional adjuvants, and combinations thereof. Paucilamellar lipid vesicles act as non-specific immune stimulators when, for example, a

vaccine is prepared by intermixing the antigen with the preformed vesicles such that the antigen remains extracellular to the vesicles. By encapsulating an antigen within the central cavity of the vesicle, the vesicle acts both as an immune stimulator and a carrier for the antigen. In another embodiment, the vesicles are primarily made of nonphospholipid vesicles. In other embodiment, the vesicles are Novasomes. Novasomes® are paucilamellar nonphospholipid vesicles ranging from about 100 nm to about 500 nm. They comprise Brij 72, cholesterol, oleic acid and squalene. Novasomes have been shown to be an effective adjuvant for antigens (*see*, U.S. Patents 5,629,021, 6,387,373, and 4,911,928, herein incorporated by reference in their entireties for all purposes).

[0096] In one aspect, an adjuvant effect is achieved by use of an agent, such as alum, used in about 0.05 to about 0.1% solution in phosphate buffered saline. Alternatively, the nanoparticles can be made as an admixture with synthetic polymers of sugars (Carbopol®) used as an about 0.25% solution. Some adjuvants, for example, certain organic molecules obtained from bacteria; act on the host rather than on the antigen. An example is muramyl dipeptide (N-acetylmuramyl-L-alanyl-D-isoglutamine [MDP]), a bacterial peptidoglycan. In other embodiments, hemocyanins and hemoerythrins may also be used. The use of hemocyanin from keyhole limpet (KLH) is preferred in certain embodiments, although other molluscan and arthropod hemocyanins and hemoerythrins may be employed.

[0097] Various polysaccharide adjuvants may also be used. For example, the use of various pneumococcal polysaccharide adjuvants on the antibody responses of mice has been described (Yin *et al.*, 1989). The doses that produce optimal responses, or that otherwise do not produce suppression, should be employed as indicated (Yin *et al.*, 1989). Polyamine varieties of polysaccharides are particularly preferred, such as chitin and chitosan, including deacetylated chitin. In another embodiment, a lipophilic disaccharide-tripeptide derivative of muramyl dipeptide which is described for use in artificial liposomes formed from phosphatidyl choline and phosphatidyl glycerol.

[0098] Other suitable adjuvants include amphipathic and surface active agents, *e.g.*, saponin and derivatives such as QS21 (Cambridge Biotech). Saponin-based adjuvants include those containing Matrix A and Matrix C, alone and in combination. Exemplary suitable saponin-based adjuvants are described in U.S. Published Application, 20120107353 and 20110081378 which are herein incorporated by reference for all purposes.

[0099] Nonionic block copolymer surfactants (Rabinovich *et al.*, 1994) may also be employed. Oligonucleotides are another useful group of adjuvants (Yamamoto *et al.*, 1988). Quil A and lentinen are other adjuvants that may be used in certain embodiments.

[00100] Another group of adjuvants are the detoxified endotoxins, such as the refined detoxified endotoxin of U.S. Pat. No. 4,866,034. These refined detoxified endotoxins are effective in producing adjuvant responses in vertebrates. Of course, the detoxified endotoxins may be combined with other adjuvants to prepare multi-adjuvant formulation. For example, combination of detoxified endotoxins with trehalose dimycolate is particularly contemplated, as described in U.S. Pat. No. 4,435,386. Combinations of detoxified endotoxins with trehalose dimycolate and endotoxic glycolipids is also contemplated (U.S. Pat. No. 4,505,899), as is combination of detoxified endotoxins with cell wall skeleton (CWS) or CWS and trehalose dimycolate, as described in U.S. Pat. Nos. 4,436,727, 4,436,728 and 4,505,900. Combinations of just CWS and trehalose dimycolate, without detoxified endotoxins, is also envisioned to be useful, as described in U.S. Pat. No. 4,520,019.

[00101] Those of skill in the art will know the different kinds of adjuvants that can be conjugated to vaccines, including alkyl lysophosphilipids (ALP); BCG; and biotin (including biotinylated derivatives) among others. Certain adjuvants particularly contemplated for use are the teichoic acids from Gram-cells. These include the lipoteichoic acids (LTA), ribitol teichoic acids (RTA) and glycerol teichoic acid (GTA). Active forms of their synthetic counterparts may also be employed. (Takada *et al.*, 1995).

[00102] Various adjuvants, even those that are not commonly used in humans, may still be employed in other vertebrates, where, for example, one desires to raise antibodies or to subsequently obtain activated T cells. The toxicity or other adverse effects that may result from either the adjuvant or the cells, *e.g.*, as may occur using non-irradiated tumor cells, is irrelevant in such circumstances.

[00103] Another method of inducing an immune response can be accomplished by formulating the nanoparticles with "immune stimulators." These are the body's own chemical messengers (cytokines) to increase the immune system's response. Immune stimulators include, but not limited to, various cytokines, lymphokines and chemokines with immunostimulatory, immunopotentiating, and pro-inflammatory activities, such as interleukins (*e.g.*, IL-1, IL-2, IL-3, IL-4, IL-12, IL-13); growth factors (*e.g.*, granulocyte-

macrophage (GM)-colony stimulating factor (CSF)); and other immunostimulatory molecules, such as macrophage inflammatory factor, Flt3 ligand, B7.1; B7.2, etc. The immunostimulatory molecules can be administered in the same formulation as the nanoparticles, or can be administered separately. Either the protein or an expression vector encoding the protein can be administered to produce an immunostimulatory effect.

[00104] Alum may be present in a range with a lower limit of: about 0.2 μg , about 0.4 μg , about 0.6 μg about 0.8 μg, about 1 μg, about 2 μg, about 3 μg, about 4 μg, about 5 μg, about 6 μg, about 7 μg, about 9 μg, about 10 μg, about 15 μg, about 20 μg, about 25 μg, about 30 μg, about 35 μg, 40 μg, about 45 μg, about 50 μg, about 60 μg, about 70μg, about 80 μg, about 90 μg, about 100 μg, about 110 μg, about 120 μg, about 130 μg, about 140 μg, or about 150 μg. Alum may be present at a range with an upper limit of: about 10 μg, about 15 μg, about 20 μg, about 25 μg, about 30 μg, about 35 μg, 40 μg, about 45 μg, about 50 μg, about 60 μg, about 70μg, about 80 μg, about 90 μg, about 100 μg, about 110 μg, about 120 μg, about 130 μg, about 140 μg, about 150 μg, or about 200 μg. In particular aspects, the alum range is about 80 μg to about 120 μg or about 100 μg to about 120 μg.

[00105] The saponin-based adjuvant may be present in a range with a lower limit of: about 0.2 μg , about 0.4 μg , about 0.6 μg about 0.8 μg, about 1 μg, about 2 μg, about 3 μg, about 4 μg, about 5 μg, about 6 μg, about 7 μg, about 9 μg, about 10 μg, about 15 μg, about 20 μg, about 25 μg, or about 30 μg. The saponin-based adjuvant may be present at a range with an upper limit of: about 10 μg, about 15 μg, about 20 μg, about 25 μg, about 30 μg, about 35 μg, 40 μg, about 45 μg, about 50 μg, about 60 μg, about 70μg, about 80 μg, about 90 μg, about 100 μg, about 110 μg, about 120 μg, about 130 μg, about 140 μg, about 150 μg, or about 200 μg. In particular aspects, the saponin-based adjuvant range is about 5 μg to about 20 μg or about 1 μg to about 10 μg.

[00106] These doses are particularly suitable in mice and may be adjusted for human use based on typical mouse weight of 20 g versus human weight of about 60 Kg.

Method of Stimulating an Anti-MERS CoV Immune Response

[00107] The nanoparticles are useful for preparing immunogenic compositions to stimulate an immune response that confers immunity or substantial immunity to MERS CoV viruses. Both mucosal and cellular immunity may contribute to immunity to infection and disease. Antibodies secreted locally in the upper respiratory tract are a major factor in resistance to

natural infection. Secretory immunoglobulin A (sIgA) is involved in protection of the upper respiratory tract and serum IgG in protection of the lower respiratory tract. The immune response induced by an infection protects against reinfection with the same virus or an antigenically similar viral strain. The antibodies produced in a host after immunization with the nanoparticles disclosed herein can also be administered to others, thereby providing passive administration in the subject.

[00108] The present disclosure provides a method of producing high affinity anti-MERS-CoV antibodies. The high affinity antibodies produced by immunization with the nanoparticles disclosed herein are produced by administering an immunogenic composition comprising a MERS-CoV nanoparticle to an animal, collecting the serum and/or plasma from the animal, and purifying the antibody from the serum/ and or plasma. In one embodiment, the animal is a human. In one embodiment, the animal is bovine or equine. In another embodiment, the bovine or equine animal is transgenic. In yet a further embodiment, the transgenic bovine or equine animal produces human antibodies. In one embodiment, the method further comprises administration of an adjuvant or immune stimulating compound. In a further embodiment, the purified high affinity antibody is administered to a human subject. In one embodiment, the human subject is at risk of MERS-CoV infection.

[00109] Nanoparticles can induce substantial immunity in a vertebrate (*e.g.* a human) when administered to said vertebrate. The substantial immunity results from an immune response against the nanoparticles that protects or ameliorates infection or at least reduces a symptom of virus infection in said vertebrate. In some instances, if the said vertebrate is infected, said infection will be asymptomatic. The response may be not a fully protective response. In this case, if said vertebrate is infected with a MERS CoV virus, the vertebrate will experience reduced symptoms or a shorter duration of symptoms compared to a non-immunized vertebrate.

[00110] In one embodiment, the disclosure provides methods of inducing substantial immunity to virus infection or at least one symptom thereof in a subject, comprising administering at least one effective dose of a nanoparticle. In another embodiment, said induction of substantial immunity reduces duration of MERS symptoms. In another embodiment, a method to induce substantial immunity to virus infection or at least one symptom thereof in a subject, comprises administering at least one effective dose of a nanoparticle. In another embodiment, said subject is a mammal. In another embodiment,

said mammal is a human. In a further embodiment, said nanoparticle is formulated with an adjuvant or immune stimulator.

[00111] In embodiments, the dose of each antigen present in a composition may be about 0.2 μg , about 0.4 μg , about 0.6 μg about 0.8 μg, about 1 μg, about 2 μg, about 3 μg, about 4 μg, about 5 μg, about 6 μg, about 7 μg, about 9 μg, about 10 μg, about 15 μg, about 20 μg, about 25 μg, about 30 μg, about 35 μg, 40 μg, about 45 μg, about 50 μg, about 60 μg, about 70μg, about 80 μg, about 90 μg, about 100 μg, about 110 μg, about 120 μg, about 130 μg, about 140 μg, or about 150 μg. For example, amounts can be measured according to MERS Spike protein content. For example, 1 μg of a nanoparticle is about 1 μg of MERS Spike protein.

Antibodies Produced by Immunization with Nanoparticles

[00112] The nanoparticles disclosed herein induce the production of high affinity anti-MERS-CoV antibodies in the host. These high affinity anti-MERS-CoV antibodies demonstrate higher affinities than anti-MERS-Co-V antibodies produced through conventional methods (e.g. phage-display; *see* Zhang et al. 2014)).

[00113] The term "affinity" refers to the strength of the interaction between an epitope and an antibody's antigen binding site. The affinity can be determined, for example, using the equation

$$K_A = \frac{[Ab\text{-}Ag]}{[Ab]\,[Ag]}$$

[00114] Where K_A = affinity constant ; [Ab] = molar concentration of unoccupied binding sites on the antibody; [Ag] = molar concentration of unoccupied binding sites on the antigen; and [Ab-Ag] = molar concentration of the antibody-antigen complex. The K_A describes how much antibody-antigen complex exists at the point when equilibrium is reached. The time taken for this to occur depends on rate of diffusion and is similar for every antibody. However, high-affinity antibodies will bind a greater amount of antigen in a shorter period of time than low-affinity antibodies. The K_A (affinity constant) of the antibodies produced can vary, and range from between about 10^5 mol^{-1} to about 10^{12} mol^{-1} or more. The K_A can be influenced by factors including pH, temperature, and buffer composition.

[00115] The antibodies produced can be polyclonal antibodies or monoclonal antibodies. The affinity of monoclonal antibodies can be measured accurately because they are

homogeneous and selective for a single epitope. Polyclonal antibodies are heterogeneous and will contain a mixture of antibodies of different affinities recognizing several epitopes – therefore only an average affinity can be determined.

[00116] The antibody affinity can be measured using any means commonly employed in the art, including but not limited to the use of biosensors, such as surface plasmon resonance (e.g. Biacore). Resonance units are proportional to the degree of binding of soluble ligand to the immobilized receptor (or soluble antibody to immobilized antigen). Determining the amount of binding at equilibrium with different known concentrations of receptor (antibody) and ligand (protein antigen) allows the calculation of equilibrium constants (K_A, K_D), and the rates of dissociation and association (koff, kon).

[00117] For example, K_D (the equilibrium dissociation constant) is a ratio of koff/kon, between the antibody and its antigen. K_D and affinity are inversely related. The lower the K_D value (lower antibody concentration), the higher the affinity of the antibody. Most antibodies have K_D values in the low micromolar (10^{-6}) to nanomolar (10^{-7} to 10^{-9}) range. High affinity antibodies are generally considered to be in the low nanomolar range (10^{-9}) with very high affinity antibodies being in the picomolar (10^{-12}) range or lower (e.g. 10^{-13} to 10^{-14} range). In one embodiment, the antibodies produced by immunization with the nanoparticles disclosed herein have a K_D ranging from about 10^{-6} to about 10^{-15}, from about 10^{-7} to about 10^{-15}, from about 10^{-8} to about 10^{-15}, and from about 10^{-9} to about 10^{-15}, from about 10^{-10} to about 10^{-15}, about 10^{-11} to about 10^{-15}, about 10^{-12} to about 10^{-15}, about 10^{-13} to about 10^{-14}, about 10^{-13} to about 10^{-15}, and about 10^{-14} to about 10^{-15}. In a preferred embodiment, the antibodies produced by immunization with the nanoparticles disclosed herein have a K_D ranging from about 10^{-10} to about 10^{-14}.

[00118] The antibodies produced by immunization with the nanoparticles disclosed herein have low rate of dissociation (K_{off}), indicating they bind tightly to the antigen. In one embodiment, the antibodies produced by immunization with the nanoparticles disclosed herein have a K_{off} ranging from about 10^{-3} M^{-1} to about 10^{-13} M^{-1}, from about 10^{-5} M^{-1} to about 10^{-13} M^{-1}, from about 10^{-6} M^{-1} to about 10^{-13} M^{-1}, from about 10^{-7} M^{-1} to about 10^{-13} M^{-1}, from about 10^{-8} M^{-1} to about 10^{-13} M^{-1}, from about 10^{-9} M^{-1} to about 10^{-13} M^{-1}, from about 10^{-10} M^{-1} to about 10^{-13} M^{-1}, from about 10^{-11} M^{-1} to about 10^{-13} M^{-1}, or from about 10^{-12} M^{-1} to about 10^{-13} M^{-1}. In another embodiment, the antibodies produced by immunization with

the nanoparticles disclosed herein have a K_{off} ranging from about 10^{-4} M^{-1} to about 10^{-10} M^{-1}, from about 10^{-4} M^{-1} to about 10^{-9} M^{-1}, or from about 10^{-4} M^{-1} to about 10^{-8} M^{-1}.

[00119] The antibodies produced by immunization with the nanoparticles disclosed herein have a high rate of association (K_{on}) indicating they bind tightly to the antigen. In one embodiment, the antibodies produced by immunization with the nanoparticles disclosed herein have a K_{on} ranging from about 10^{3} M to about 10^{8} M, from about 10^{4} M to about 10^{7} M, from about 10^{4} M to about 10^{6} M, from about 10^{4} M to about 10^{5} M, from about 10^{5} M to about 10^{7} M, or from about 10^{5} M to about 10^{6} M.

[00120] The antibodies produced by immunization with the nanoparticles disclosed herein neutralize the MERS-CoV virus. The antibodies can be broadly neutralizing antibodies and neutralize one or more MERS-CoV virus strains, clades, or coronaviruses, or the antibodies can neutralize only one MERS-CoV virus strain. The neutralization ability of the antibodies can be measured by any means commonly employed in the art, including, but not limited to, Fluorescence Reduction Neutralization Test ($FRNT_{50}$), in vitro cell-based assays which measure the amount of virion released from immunized cells, and in vivo assays which measure the infection in an immunized animal.

[00121] This disclosure is further illustrated by the following examples which should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application, as well as the Figures, are incorporated by reference in their entirety for all purposes.

EXAMPLE 1

Production of MERS CoV Spike nanoparticles

[00122] The nanoparticles are produced by over-expressing the MERS CoV Spike protein (FIGS. 8 and 9) in baculovirus in Sf9 cells. Following purification the nanoparticle VLPs containing Spike protein was recovered predominantly in trimer form (FIGS. 4 and 6).

EXAMPLE 2

Induction of Immune Response Using MERS CoV Spike Nanoparticles

[00123] The VLPs were administered to mice as shown in Figure 5. At the end of the 45 day period, blood was extracted from the mice to assess the immune response. Figure 7 illustrates neutralizing antibody titres for mice administered either the SARS CoV Spike nanoparticle VLP or the MERS CoV Spike nanoparticle VLP. The highest response was obtained by using the adjuvant Matrix M1.

EXAMPLE 3

Potent Anti-MERS-CoV Human Immunoglobulin Produced From Transchromosomic Bovines Inhibits MERS-CoV In Vivo

[00124] To demonstrate that anti-MERS CoV hIgG antibodies and immunoglobulin obtained from Tc bovines after vaccination could neutralize MERS CoV *in vitro* and *in vivo* we administered three experimental MERS CoV vaccines to three separate groups of Tc Bovines to induce high titer neutralizing antibodies to MERS CoV. The first vaccine tested was a whole killed Jordan strain (clade A) MERS-CoV virion (WKV) vaccine, the second was an Al-Hasa (clade B) MERS-CoV Spike nanoparticle (SN) vaccine, and the third was an Al-Hasa MERS-CoV Spike protein pDNA vaccine. The pDNA vaccine was found to be poorly immunogenic by *in vitro* assay and was not evaluated further (data not presented). However convalescent serum and highly purified hIgG immunoglobulin from both the WKV (termed SAB-300) and SN (termed SAB-301) vaccinated Tc bovines were able to cross neutralize virus *in vitro*, do not induce antibody dependent entry of MERS-CoV into human Raji B cells, and rapidly reduce viral infection (Erasmus strain) in a Ad5-hDPP4 transduced mouse model of MERS-CoV.

METHODS AND MATERIALS

ANIMAL STUDIES

Transcromosomic (Tc) Bovine Cloning and Antibody Production

[00125] Tc bovines were produced as described in Matsushita, *et al.* PLoS One, 2014. 9(3): p. e90383, Sano, *et al.* PLoS One, 2013. 8(10): p. e78119, and Kuroiwa, *et al.*, Nat Biotechnol, 2009. 27(2): p. 173-81. Briefly, the Tc bovines used in this study are homozygous for triple knock-outs in the endogenous bovine immunoglobulin genes (*IGHM*$^{-/-}$ *IGHML1*$^{-/-}$ *IGL*$^{-/-}$) and carry a human artificial chromosome (HAC) consisting of a human

chromosome 14 fragment, which contains the entire human immunoglobulin heavy chain locus, and a human chromosome 2 fragment, which contains the entire human immunoglobulin kappa light chain locus.

Inactivated whole virion production

[00126] Whole inactivated MERS-CoV virions were produced for vaccination by collecting media from Vero CCL-81 cells infected with the Jordan-N3/2012 strain (GenBank KC776174.1). $4x10^8$ PFU of MERS-CoV was irradiated with a cobalt source until a dose of 6MRads was achieved. The material was safety tested for inactivation. No MERS-CoV specific signal was detected in the irradiated passaged material.

MERS-CoV Spike Nanoparticle production

[00127] Purified Al-Hasa strain MERS-CoV spike protein nanoparticles were produced as described in Coleman *et al.* 2014. Briefly Sf9 cells at 2-3 x 10^6 cells/ml were infected with specific recombinant baculovirus. Infected Sf9 cells were incubated with continuous agitation at 27 $\pm$ 2 °C and harvested at 68-72 hpi by centrifugation at 4000xg for 15 minutes. Spike proteins were extracted from cellular membranes with a non-ionic detergent and insoluble material was removed by centrifugation at 10,000xg for 30 minutes. Spike protein assemblies were purified using a combination of anion exchange, affinity, and size exclusion chromatography. During purification, the majority of the detergent is removed, allowing Spike protein trimers to form higher ordered protein-protein micellular nanoparticles. Purified Spike nanoparticles were 0.2 micron filtered and stored at -80 °C.

Tc Bovine vaccination

[00128] As shown in Figure 10A, three Tc bovines in Group 1 (#2244; #2252 & #2254) were immunized with whole killed MERS-CoV virus (WKV) at 1-2 x 10^8 PPF/dose formulated with Montanide ISA 25 adjuvant (Seppic) as oil-in-water emulsion plus the saponin-derived immune stimulant Quil A (Accurate Chemicals). Two Tc bovines in Group 2 (#2178 & #2183) were immunized with recombinant MERS-CoV Spike Nanoparticle (SN) at 2 mg/dose formulated with Montanide ISA 206 adjuvant as water-in-oil-in-water emulsion plus Quil A. The Tc bovines in both groups were immunized 5 times at three- to four-week intervals.

In vivo MERS-CoV Challenge studies in Adenovirus/hDPP4 mice

[00129] Transduced hDPP4 BALB/c mice were infected with the Erasmus strain MERS-CoV. Briefly, BALB/c were injected intraperitoneally with 100 or 500 μg of negative control or test immunoglobulin collected from the bovines (SAB-300 or SAB-301). Twelve hours later, mice were infected intranasally with MERS-CoV ($1x10^5$ PFU) in a total volume of 50 μl. To obtain virus titers, lungs were removed into PBS and homogenized using a manual homogenizer. Virus was titered on Vero 81 cells. Viral titers are expressed as PFU/g tissue for MERS-CoV.

RESULTS AND DISCUSSION

Evaluation of antigen-specific hIgG produced in Tc animals

[00130] MERS-CoV Spike protein specific ELISAs were performed to evaluate the antigen-specific hIgG response in Tc bovines following the immunization with WKV or SN (Figure 10B) and SAB-300 and SAB-301 (Figure 10C). The ELISA results demonstrated robust MERS-CoV spike–specific hIgG response at the second vaccination (V2) and maintained high titers through the last immunization (V5) for both the serum and SAB-300 and SAB-301 (Figure 10). This data indicate that high titer hIgG antibodies targeting MERS-CoV proteins were generated from the Tc bovines immunized with WKV or SN and that both immunoglobulins retained activity after purification.

In Vitro Neutralization of MERS-CoV by Sera, SAB-300 and SAB-301

Fluorescence Reduction Neutralizing Test-50% reduction ($FRNT_{50}$).

[00131] Two neutralization assays were performed to identify the neutralization potential of SAB-300 and SAB-301 *in vitro*. Sera from vaccinated bovine, SAB-300, and SAB-301 were assayed for their ability to inhibit infection of MERS-CoV *in vitro* using a Fluorescence Reduction Neutralizing Test ($FRNT_{50}$) and a viral infection neutralization assay. The FRNT assay reports infection using fluorescence and the viral infection neutralization assay reports using cell viability.

[00132] The $FRNT_{50}$ assay was performed on sera from vaccinated bovines (Figure 11A), and SAB-300 and SAB-301 (Figure 11B). We find that bovine #2244 V2 produced high titer neutralizing antibody as did V2 from bovines #2178 and #2183. When compared to an equal

amount of the positive control Anti-Spike rabbit antibody that is antigen affinity purified, Tc bovine V2 sera are significantly more neutralizing. This data is consistent with the antigen-specific ELISA results in Figure 10B. SAB-300, purified hIgG from bovine #2244 V2, and SAB-301, purified hIgG from V2 of #2178 and #2183, were then tested for neutralization in the same $FRNT_{50}$ assay. The data demonstrate that SAB-300 and SAB-301 generated similar high levels of neutralizing antibody titers (Figure 11C). Compared to the equal amounts of a positive control antigen affinity purified a rabbit Anti-Spike antibody, both purified hIgG preparations were highly effective at neutralizing MERS-CoV demonstrating that both SAB-300 and SAB-301 were able to inhibit infection of MERS-CoV *in vitro*.

[00133] Additionally, SAB-300 and SAB-301 caused significant neutralization of MERS-CoV grown in Vero E6 cells, whereas the no-specific control serum had no significant effect on MERS-CoV infection of Vero E6 cells (Figure 12A). Levels of infectious MERS-CoV released from cells infected with MERS-CoV pre-treated with SAB-300 were below the detection limit of the assay (158 $TCID_{50}$/ml; Figure 12A) indicating that SAB-300 is a very strong neutralizing antibody. Levels of infectious MERS-CoV released from cells infected with MERS-CoV pre-treated with SAB-301 were below the detection limit of the assay (158 $TCID_{50}$/ml; Figure 12A) at all dilutions except 1:8000 and 1:16000 suggesting that serum SAB-301 is a strong neutralizing antibody, but not as inhibitory as SAB-300.

SAB-300 and SAB-301 do not cause antibody enhancement of MERS-CoV

[00134] To demonstrate that MERS-CoV viruses bound by antibodies do not allow for entry of the virion into cells that are normally not infected, Raji cells, an immortalized human B cell line that is not typically infected by MERS-CoV, were tested to see if the presence of anti-MERS antibodies could allow for transcription of viral RNA and release of live virus. Raji cells were infected with MERS-CoV pre-incubated with SAB-300 and SAB-301 (Figure 12B). RT-PCR for viral mRNA and $TCID_{50}$ assays were performed on the media at 48 hours post infection to assess infection. The TCID50 assay could not detect MERS-CoV released from the Raji cells indicating they were not infected by MERS-CoV. Conversely, this assay found that similarly treated Vero E6 cells, which are typically infected by MERS-CoV, produce high levels of virus at 48 hours post infection (data not shown). To determine if MERS-CoV RNA was detected in Raji cells infected with MERS-CoV after pre-incubation with SAB-300 and SAB-301. RNA was extracted from infected cells and analyzed by

Taqman realtime PCR with primers specific to MERS-CoV newly transcribed RNA (leader primer) (Figure 12B). There was no significant detection of MERS-CoV newly transcribed viral RNA (leader primer set) in Raji cells infected with MERS-CoV alone, infected with the non-specific negative control hIgG purified from pre-vaccination plasma, or with cells infected with MERS-CoV pre-treated with SAB-300 or SAB-301. These data demonstrate that there is no antibody dependent enhancement of MERS-CoV infection caused by SAB-300 or SAB-301.

Inhibition of MERS-CoV replication in vivo

[00135] We tested the efficacy of SAB-300 and SAB-301 in a mouse model of MERS-CoV. Mice are non-permissive to MERS-CoV; however, when transduced with an adenovirus expressing the MERS-CoV receptor, human Dipeptidyl peptidase 4 (hDPP4), they become permissive and replicate virus (Zhao, *et al.*, Proc Natl Acad Sci U S A, 2014. 111(13): p. 4970-5). To test the anti-viral activity of the Tc antibodies, BALB/c mice (6-8 weeks) were transduced intranasally with $2.5x10^8$ PFU of Ad5-hDPP4 in 75ul PBS. At 5 days post transduction, mice were treated by intraperitoneal injection with a single 100 or 500μg dose of control hIgG, SAB-300 or SAB-301. Twelve hours later, mice were infected intranasally with MERS-CoV ($1x10^5$ PFU) in a total volume of 50μl. No additional antibody injections were given over the 5-day course of the infection. At 1, 3 and 5 days post infection mice were euthanized and their lungs were dissected. To obtain virus titers, lungs were homogenized in PBS using a manual homogenizer, clarified by centrifugation and titered on Vero cells (Figure 13). Titers of ~$1x10^6$ PFU/mg of lung tissue were found in the lungs of the no treatment group, and the group receiving negative control human IgG at days 1 and 3 post infection with titers dropping to $1x10^5$ at 5dpi. Mice injected with either 100μg or 500μg of SAB-300, ~50-fold or 500-fold reductions, respectively, had virus titers was found at day 1 post infection compared to the hIgG control injection group (Figure 13A). By day 3 post infection, the reduction in virus titer for the 100μg group was ~5000-fold while titers in the mice receiving 500ug were below the level of detection. At 5 days post infection, virus titers in both treated groups were below the level of detection (Figure 13A). Similar reductions in virus titer were found for the SAB-301 antibody, except that lung titers were modestly higher at day 1 post infection compared to the SAB-300 treated mice (Figure 13B). By day 5 post infection, all SAB-301 treated mice had virus titers below the level of detection

(Figure 13B). These data demonstrate that both SAB-300 and SAB-301 were able to protect mice from MERS-CoV infection with a single prophylactic injection.

EXAMPLE 4

BIACORE Analysis of antibodies in animals immunized with MERS-CoV Spike protein (S) nanoparticles

[00136] The avidity of two antibodies from two Tc bovine (#2178 and 2183) immunized with the MERS-CoV S nanoparticle vaccine following a series of immunizations as shown in Figure 10A and Example 3 was tested by anti-S affinity (Biacore™) analysis. We tested the avidity of antibodies in the serum of these animals after vaccination 3 (v3), vaccination 4(v4), and vaccination 5 (v5). Briefly, using a protein A/G amine coupled to a CM5 chip, the bovine Tc serum IgG was captured to the flow cell. MERS Spike protein antigen at 0, 20, and 40 nM was injected to the flow cell for 180 seconds and followed by disassociation for 600 seconds. The 1:1 fit model was applied. Association and dissociation rates were measured as a function of time and plotted on a sensorgram, and the dissociation constant was calculated from the association and dissociation rates. The results are shown in Figure 14 and Table 1.

Table 1

Binding kinetics of MERS-CoV S antigen to Tc human IgG from bovines			
serum samples	k_{on} (Ms)	k_{off} (/s)	K_D (M)
2178 v3	6019	$5.69x10^{-4}$	$9.45x10^{-8}$
2178 v4	$1.44x10^{5}$	$1.79x10^{-7}$	$1.24x10^{-12}$
2178 v5	$9.80x10^{4}$	$1.23x10^{-7}$	$1.25x10^{-12}$
2183 v3	$7.97x10^{4}$	$3.90x10^{-7}$	$4.89x10^{-12}$
2183 v4	$1.07x10^{5}$	$1.04x10^{-8}$	$9.78x10^{-14}$
2183 v5	$1.10x10^{5}$	$1.71x10^{-8}$	$1.57x10^{-13}$

[00137] Table 2 shows the affinity of anti-S1 mAbs produced by phage-display as disclosed in Ying *et al.* J. Virol. (2014). A comparison of the data in these two tables demonstrates that the reported K_D and antibody K_{off} rates of the MERS-CoV mAbs produced through phage

display are orders of magnitude less than the polyclonal anti-S responses induced in the Tc bovine.

Table 2

Binding kinetics of mAb to MERS-CoV RBD (Ying et al. (2014))			
mAb	k_{on} (Ms)	k_{off} (/s)	K_D (M)
m336	$1.66x10^{6}$	$1.65x10^{-4}$	$9.94x10^{-11}$
m337	$1.87x10^{5}$	$1.54x10^{-4}$	$8.24x10^{-10}$
m338	$3.55x10^{5}$	$1.98x10^{-4}$	$5.58x10^{-10}$

OTHER EMBODIMENTS

[00138] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments described herein. Such equivalents are intended to be encompassed by the claims provided herein.

INCORPORATION BY REFERENCE

[00139] This application incorporates by reference all publications or references disclosed herein for all purposes in their entireties.

[00140] This application incorporates by reference for all purposes the entirety of the following: U.S Serial No. 12/306,965, filed June 27, 2007, U.S. Serial No. 61/015,440 filed on December 20, 2007, U.S. Serial No. 11/582,540, filed October 18, 2006, U.S. Serial No. 12/633,995, filed October 18, 2006 U.S. Serial Nos. 60/727,513, filed October 18, 2005; 60/780,847, filed March 10, 2006; 60/800,006, filed May 15, 2006; 60/831,196, filed July 17, 2006; 60/832,116, filed July 21, 2006, and 60/845,495, filed September 19, 2006, and 10/617,569, filed July 11, 2003.

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We Claim:

1. An immunogenic composition comprising a MERS-CoV nanoparticle, wherein the nanoparticle comprises at least one trimer of a Spike polypeptide.

2. The composition of claim 1, wherein the nanoparticle comprises at least about five trimers to about 30 trimers of the Spike polypeptide.

3. The composition of claim 2, wherein the nanoparticle comprises at least about ten trimers to about 20 trimers of the Spike polypeptide.

4. The composition of claim 1, wherein the concentration of the nanoparticle is at least about 20 μg/mL to about 60 μg/mL.

5. The composition of claim 4, wherein the concentration of the nanoparticle is at least about 30 μg/mL to about 50 μg/mL.

6. The composition of any of claims 1 to 3, wherein the Spike polypeptide comprises SEQ ID NO: 2.

7. The composition of any of claims 1 to 3, wherein the Spike polypeptide consists of SEQ ID NO: 2.

8. A method of producing a high affinity antibody comprising administering to an animal an immunogenic composition comprising a MERS-CoV nanoparticle, wherein the nanoparticle comprises at least one trimer of a Spike polypeptide, collecting serum and/or plasma from the animal, and purifying the antibody from the serum and/or plasma.

9. The composition of claim 8, wherein the nanoparticle comprises at least about five trimers to about 30 trimers of the Spike polypeptide.

10. The composition of claim 9, wherein the nanoparticle comprises at least about ten trimers to about 20 trimers of the Spike polypeptide.

11. The composition of claim 8, wherein the concentration of the nanoparticle is at least about 20 μg/mL to about 60 μg/mL.

12. The composition of claim 11 wherein the concentration of the nanoparticle is at least about 30 μg/mL to about 50 μg/mL.

13. The composition of any of claims 8 to 10, wherein the Spike polypeptide comprises SEQ ID NO: 2.

14. The composition of any of claims 8 to 10, wherein the Spike polypeptide consists of SEQ ID NO: 2.

15. The method of claim 8, further comprising the administration of an adjuvant.

16. The method of claim 15, wherein the adjuvant is selected from the group consisting of Montanide ISA 206, Quil A, Alum, Freund's adjuvant, incomplete Freund's adjuvant, and aluminum hydroxide adjuvants.

17. The method of claim 8, wherein the animal is bovine or equine.

18. The method of claim 17, where the bovine or equine animal is a transgenic animal.

19. The method of claim 8, wherein the antibody has a KD between 10^{-8} and 10^{-15}.

20. The method of claim 19, wherein the antibody has a KD between 10^{-12} and 10^{-14}.

21. The method of claim 8, further comprising administering the purified high affinity antibody to a human subject.