SARS VACCINES AND METHODS TO PRODUCE HIGHLY POTENT ANTIBODIES

This application claims benefit of U.S. Serial No. Not Yet Known, Filed May 31, 2005 and U.S. Serial No. 60/576,118, filed June 2, 2004, which is incorporated in its entirety by reference into this application.

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

BACKGROUND OF THE INVENTION

Severe acute respiratory syndrome (SARS), a newly emerging infectious disease, is caused by a SARS-associated coronavirus (SARS-CoV) (1-7), which may originate from some wild animals (8). A global outbreak of SARS in 2002/2003 resulted in thousands of cases and hundreds of deaths, seriously threatening public health worldwide. In late 2003 and early 2004, new infections caused by SARS-CoV strains different from those predominant in 2002/2003 epidemic were reported in China (9). Several isolated outbreaks that resulted from accidental releases of the SARS-CoV isolates were reported in Taiwan, Singapore, and China (http://www.who.int/csr/sars/en). These indicate that SARS epidemics may recur at any time in the future, either by animal-to-human transmission of the SARS-CoV or by the virus escaping from laboratory samples. Therefore, development of effective and safe vaccines is urgently needed for protection of at-risk populations.

Currently, one candidate vaccine using inactivated SARS-CoV is in a phase I clinical trial in China (9, 10). Although the

inactivated SARS-CoV has been shown to be effective in protecting animals from challenge by SARS-CoV, its efficacy in humans is unclear. There has been a serious concern about its safety since some antigens in the virions may elicit antibodies that do not neutralize, but rather enhance virus infection (10). Some viral proteins may induce harmful immune and inflammatory responses, a potential cause of SARS pathogenesis and the rationale for using immunosuppressants (e.g., steroids) for SARS treatment, although there are apparent contradictions to this regimen (11, 12). Most recently, it was reported that SARS-CoV infection of ferrets caused mild liver inflammation and the liver damage became much more serious if the ferrets were first immunized with vaccinia virus-based SARS vaccines before virus challenge (13).

The S proteins of coronaviruses are responsible for virus binding, fusion and entry, and are major inducers of neutralizing antibodies (14-16). Besides, they play critical roles in viral pathogenesis and virulence (17). The S protein of SARS-CoV is also important for viral functions and antigenicity (18, 19). It is a type I transmemberane glycoprotein consisting of two domains, S1 and S2 (18) (Fig. 1). S1 is responsible for virus binding to the receptor on the target cell. It has been demonstrated that angiotensin-converting enzyme 2 (ACE2) is a functional receptor for SARS-CoV (20-23). A fragment located in the middle region of S1 is the receptor-binding domain (RBD)(24-26). S2 domain, which contains a putative fusion peptide and two heptad repeat (HR1 and HR2) regions (Fig. 1), is responsible for fusion between viral and target cell membranes. Like the anti-HIV peptides derived from the HIV-1 gp41 HR2 region (27, 28), a peptide derived from the HR2 region of SARS-CoV S protein was identified to be an inhibitor of SARS-CoV infection (29). HR1 and HR2 regions can associate to form a six-helix bundle structure (29, 30), resembling the fusion-active core structure of gp41 in HIV (31)

PCT/US05/19266

and of the S proteins in other coronavirus, such as mouse hepatitis coronavirus (MHV)(32, 33). These suggest that upon binding of RBD on the viral S protein to ACE2 on the target cell, S2 changes conformation by interaction between the HR1 and HR2 regions to form fusogenic core and bring viral and target cell membrane into close proximity, resulting in virus fusion and entry (29). This indicates that the fragments containing the functional domains on the S protein may be used as antigens for inducing antibodies to block virus binding or fusion.

Several live attenuated and genetically engineered vaccines encoding SARS-CoV S protein have been in preclinical studies. Recently, Nabel and colleagues (34) reported that a DNA vaccine candidate encoding the S protein induced T-cell and neutralizing-antibody responses (neutralizing antibody titers range from 1:50 to 1:150), and protected mice from SARS-CoV challenge as shown by reduced titers of SARS-CoV in the respiratory tracts. They proved that the protection was mediated by neutralizing antibodies but not a T-cell-dependent mechanism. Most recently, Moss and co-workers (35) demonstrated that intranasal or intramuscular inoculations of mice with highly attenuated modified vaccinia virus vectors virus Ankara (MVA) containing the gene encoding full-length SARS-CoV S protein (MVA/S) produced S-specific antibodies with SARS-CoV neutralizing activity (mean neutralizing titer is 1:284), and protected mice from SARS-CoV infection after transfer of serum from immunized mice. These data suggest that the S protein can induce protective neutralizing antibodies, although the neutralizing antibody titers are relatively low.

A recombinant fusion protein containing RBD linked to a human IgG-Fc fragment (for facilitating RBD purification) as an antigen (designated RBD-Fc, see Fig. 1) for immunization of mice and rabbits can induce highly potent neutralizing antibody responses in the immunized animals (geometric mean neutralizing

PCT/US05/19266

titer 1:>10,000) and the antibodies can bind to RBD and block RBD binding to ACE2. This suggests that RBD may be applied as a subunit vaccine for prevention of SARS.

This invention discloses a recombinant fusion protein or isolated polypeptides containing RBD linked to a human IgG-Fc fragment (for facilitating RBD purification) as an antigen (designated RBD-Fc, see Figure 1) that can induce highly potent neutralizing antibody responses in immunized animals (mean neutralizing titer 1:15,360 for rabbits and 1:12,553 for mice), suggesting that RBD may be applied as a subunit vaccine for prevention of SARS.

PCT/US2005/019266

SUMMARY OF THE INVENTION

The spike (S) protein of severe acute respiratory syndrome (SARS) coronavirus (CoV), a type I transmembrane envelope glycoprotein, consists of S1 and S2 domains responsible for virus binding and fusion, respectively. The S1 contains a receptor-binding domain (RBD) that can specifically bind to angiotensin-converting enzyme (ACE2), the receptor on target cells.

This invention provides a vaccine comprising an effective amount of the isolated polypeptide or recombinant protein containing the sequence of RBD in the Severe Acute Respiratory Syndrome (SARS) associated coronavirus spike protein or a functional fragment thereof.

This invention also provides a vaccine comprising an effective amount of a nucleic acid molecule comprising the sequence of a fragment which encodes the sequence of RBD in the Severe Acute Respiratory Syndrome associated coronavirus spike protein, or a functional fragment thereof.

This invention also provides a recombinant fusion protein containing sequence of RBD in the Severe Acute Respiratory Syndrome associated coronavirus spike protein, or a functional fragment thereof, and sequence of a human IgG Fc fragment (designated RBD-Fc), or a functional fragment thereof.

RBD-Fc can induce highly potent antibody responses in the immunized animals, including rabbits and mice. The antibodies recognized the sequence of RBD on S1 domain in the Severe Acute Respiratory Syndrome associated coronavirus spike protein, the sequence of S1 domain in the Severe Acute Respiratory Syndrome associated coronavirus spike protein, and the sequence of the

PCT/US05/019266

Severe Acute Respiratory Syndrome associated coronavirus spike protein.

The antibodies from animals (e.g., rabbits and mice) immunized by RBD-Fc effectively blocked binding of RBD or S1 domain in the Severe Acute Respiratory Syndrome associated coronavirus spike protein to soluble ACE2 molecules or ACE2 expressed on cells.

The antibodies from animals (e.g., rabbits and mice) immunized by RBD-Fc potently neutralized infection by SARS-CoV and by HIV/SARS-CoV S pseudovirus with a neutralizing titer about 50-300-fold higher than those of the mouse antisera induced by DNA vaccines and vaccinia virus vectors encoding the full-length of SARS-CoV S protein.

Depletion of anti-Fc antibodies from antisera did not affect neutralizing activity.

This indicates that the sequence of RBD on S1 domain in the Severe Acute Respiratory Syndrome associated coronavirus spike protein can induce highly potent neutralizing antibody responses and can be developed as an effective and safe subunit vaccine for prevention of SARS.

The IgG Fc linked to RBD may significantly enhance the immunogenicity of RBD to produce high levels of specific antibodies against RBD. The method of linking IgG Fc to an antigen may be used for inducing high levels of antibodies against the corresponding antigen.

This invention provides a composition for increasing the immunogenicity of an antigen comprising an effective amount of an antigen and an IgG Fc domain, its functional fragment, or a substance containing an IgG Fc domain or its functional fragment. In an embodiment, the antigen and the IgG Fc are

PCT/US2005/019266

linked. In a further embodiment, they are linked to form a fusion protein.

Finally, this invention also provides methods for using any of the above compositions for immunization. In an embodiment, they are used as vaccines.

DETAILED DESCRIPTION OF THE FIGURES

Figure 1. Schematic diagram of SARS-CoV S protein and the recombinant fusion protein RBD-Fc. The S protein consists of S1 and S2 domains. There is a signal peptide (SP) located at the N-terminus of the S protein. The S1 domain contains a receptor-binding domain (RBD). The S2 domain contains a cytoplasm domain (CP), a transmembrane domain (TM) and an ectodomain composed of a putative internal fusion peptide (FP) and heptad repeat 1 and 2 (HR1 and HR2) regions. RBD-Fc consists of RBD and a human IgG-Fc fragment. S1-C9 contains S protein S1 domain and a C9 fragment.

Figure 2. Mouse antisera contained high titers of antibodies binding to RBD on SARS-CoV S protein S1 domain. (A) Binding to RBD-Fc by antisera (1:10,000) collected from mice before immunization (pre-immune) and 4 days after each boost; (B) Binding to RBD-Fc by mouse antisera collected 4 days after the third boost at a series of 5-fold dilutions; and (C) Binding to S1-C9 protein by mouse antisera collected 4 days after the third boost at a series of 5-fold dilutions. All samples were tested in duplicate and data presented are mean values of two tests (same for the following figures).

Figure 3. Rabbit antisera contained high titers of antibodies binding to RBD. (A) Binding to RBD-Fc by antisera (1:10,000) collected from rabbits before immunization (pre-immune) and 10 days after each boost; (B) Binding to RBD-Fc by rabbit antisera collected 10 days after the first boost at a series of 5-fold dilutions; and (C) Binding to S1-C9 protein by rabbit antisera collected 10 days after the first boost at a series of 5-fold dilutions.

Figure 4. Neutralization of SARS-CoV infection by mouse antisera directed against RBD-Fc. (A) Inhibition of CPE induced by SARS-

PCT/US2005/019266

CoV replication in Vero E6 cell monolayer by mouse antisera in a series of 2-fold dilutions was quantitated. The results obtained from the experiment using antiserum from the mouse M8 was shown here as an example. The CPE was recorded under a microscope and the virus-neutralizing titers were calculated; and (B) Neutralization of HIV/SARS-CoV S pseudovirus infection by mouse antisera at a series of 2-fold dilutions. Inhibition of a single-cycle infection of 293T cells expressing ACE2 by the pseudovirus was determined in a luciferase assay.

Figure 5. Inhibition of CPE induced by SARS-CoV infection in Vero E6 monolayer by rabbit antisera was detected as described in Fig. 4A.

Figure 6. Neutralization of HIV/SARS-CoV S pseudovirus infection by rabbit antisera. Inhibition of a single-cycle infection of 293T cells expressing ACE2 by the pseudovirus was determined in a luciferase assay.

Figure 7. Effect of depletion of anti-Fc antibodies from the rabbit antisera on binding to S1-C9 and virus-neutralizing activity. The binding activity of anti-Fc-depleted and untreated rabbit antisera to human IgG (A) and S1-C9 (B) was measured by ELISA. The neutralizing activity of the anti-Fc-depleted rabbit antisera against HIV/SARS-CoV S pseudovirus was compared with that of untreated rabbit antisera (C).

Figure 8. Mouse and rabbit antisera blocked binding of S1 which contains RBD to ACE2. Inhibition of S1-C9 binding to soluble ACE2 by mouse (A) and rabbit (B) antisera was measured by ELISA. Inhibition of S1-C9 binding to cell-expressed ACE2 by rabbit antisera was measured by flow cytometry (C). In the positive control, no rabbit serum was added while in the negative control, neither rabbit serum nor S1-C9 was added. Rabbit

antisera inhibited S1-C9 binding to ACE2-expressing cells in a dose-dependent manner (D).

DETAILED DESCRIPTION OF THE INVENTION

This invention provides a vaccine comprising an effective amount of the isolated polypeptide or recombinant protein containing the receptor-binding domain (RBD) in the Severe Acute Respiratory Syndrome associated coronavirus spike protein or a functional fragment thereof. In an embodiment, appropriate adjuvant(s) is/are used with the said vaccines which are described in this invention. In a further embodiment, the vaccines are conjugated.

As used herein, functional fragment is the part of the RBD which carries out the function. In an embodiment, the function is to bind receptors.

Peptide or Polypeptide or protein with RBD Sequence

This invention provides an isolated peptide or polypeptide or protein comprising sequence of receptor-binding domain in the Severe Acute Respiratory Syndrome associated coronavirus spike protein or a functional fragment thereof, which can be used as a vaccine for preventing infection by Severe Acute Respiratory Syndrome associated coronavirus.

In an embodiment, the RBD is having the below sequence:

NITNLCPFGEVFNATKFPSVYAWERKKISNCVADYSVLYNSTFFS
TFKCYGVSATKLNDLCFSNVYADSFVVKGDDVRQIAPGQTGVIA
DYNYKLPDDFMGCVLAWNTRNIDATSTGNYNYKYRYLRHGKLRPFERDIS
NVPFSPDGKPCTPPALNCYWPLNDYGFYTTTGIGYQPYRVVVLSFELLNAP
ATV. (SEQ ID NO: 1)

This invention is intended to cover the below sequence or a functional fragment of the below sequence:

NITNLCPFGEVFNATKFPSVYAWERKKISNCVADYSVLYNSTFFS
TFKCYGVSATKLNDLCFSNVYADSFVVKGDDVRQIAPGQTGVIA
DYNYKLPDDFMGCVLAWNTRNIDATSTGNYNYKYRYLRHGKLRPFERDIS
NVPFSPDGKPCTPPALNCYWPLNDYGFYTTTGIGYQPYRVVVLSFELLNAP
ATV. (SEQ ID NO: 1)

This invention is intended to cover the RBD sequences with natural mutations in the SARS-CoV strains identified so far (6): such as R344 → K; F360 → S; L472 → P; N479 → K; D480 → G; T487 → S, and any unnatural mutations.

As it is known in this art, mutation, substitution, insertion and deletion of the sequences are possible while the RBD function or immunogenicity remains unchanged. It is the intention of this invention to include said mutation, substitution, insertion and deletion.

Nucleic Acid Fragment Encoding RBD Sequence

Nucleic acid vaccines offer a new opportunity to immunize with materials that are entirely gene-based, expressed by the recipient's own cells. There is greater control over the immunization process. The vaccine may be administered in skin or muscle. Other molecules such as cytokines may be co-expressed. In addition immunostimulatory DNA sequences may be used to modulate the type of response (Th1 or Th2). The duration of the response can be controlled by repeated exposure to the genes, which are expressed transiently, by a variety of delivery mechanisms such as: direct injection; electroporation; mucosal delivery, etc. The ability to make DNA molecules strictly by rational design makes it possible to bypass years of development for the production of efficacious vaccines. These vaccines are expressed and presented in the host, making an ideal mimic of intracellular antigens. See U.S. Patent Nos. 6,339,068B1; 6,821,957B2.

A DNA molecule comprising a nucleic acid fragment (e.g, DNA vaccine) encoding sequence of receptor-binding domain in the Severe Acute Respiratory Syndrome associated coronavirus spike protein or a functional fragment thereof, can be used as a vaccine for preventing infection by Severe Acute Respiratory Syndrome associated coronavirus.

Live attenuated viruses (e.g., MVA) containing vectors comprising the nucleic acid fragments or all molecules which comprise the sequence of said fragments encoding sequence of receptor-binding domain in the Severe Acute Respiratory Syndrome associated coronavirus spike protein or a functional fragment thereof, can be used as a vaccine for preventing infection by Severe Acute Respiratory Syndrome associated coronavirus.

Fusion Protein or Polypeptide Containing RBD Sequence Linked with IgG Fc Domain

A fusion protein or an isolated polypeptide that contain sequence of receptor-binding domain in the Severe Acute Respiratory Syndrome associated coronavirus spike protein or a functional fragment thereof, linked to a substance comprising an IgG Fc domain, its functional fragment or a substance containing an IgG Fc domain or its functional fragment, can be used as a vaccine for preventing infection by Severe Acute Respiratory Syndrome associated coronavirus

An IgG molecule can be cleaved by the enzyme papain with the hinge region at a site upstream of the inter-H chain disulfide bonds to produce two Fab fragments and one Fc fragment (59). One of the main functions of the Fc domain is responsible for binding of IgG molecule to Fc receptors (FcγR) on cell surfaces (60).

An example of the IgG sequence is illustrated below:

THTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPQVKFNWYVDGV
QVHNAKTKPREQQYNSTYRVVSVLTVLHQNWLDGKEYKCKVSNKALPAPIEKTISKAKGQ
PREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDG
SFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG. (SEQ ID NO: 2)
(61)

IgG Fc from different species should work the same.

This invention provides a pharmaceutical composition comprising any of the above described isolated polypeptide or any of the above-described the fusion protein and a pharmaceutically acceptable carrier.

This invention provides a method for induction of antibodies against Severe Acute Respiratory Syndrome-associated Coronavirus in a subject comprising administering to the subject any of the above vaccine or an effective amount of the isolated polypeptide of any of the above described fusion protein or the composition.

In an embodiment, the induced antibodies are neutralizing.

As stated herein, subjects are organisms which have immune response. The subject includes but is not limited to mammalians. Said subject includes human but could be animals, such as dogs and cats.

The above method may produce polyclonal or monoclonal antibody.

This invention provides the antibody generated by any of the above methods. These antibodies may be used to treat or prevent infection by Severe Acute Respiratory Syndrome-associated Coronavirus.

Anti-idiotypic Antibody

This invention provides an anti-idiotypic antibody which should mimic the RBD, or a functional portion thereof, against the monoclonal antibody specific to RBD of SARS corona virus.

This invention also provides a vaccine comprising an effective amount of the anti-idiotypic antibody, a functional portion thereof or a single chain antibody which can function like the anti-idiotypic antibody.

This invention provides a method for determining the neutralizing epitope contained in S1 of the Severe Acute Respiratory Syndrome Virus comprising steps of:

(a) generating peptide from the RBD sequence of SARS-CoV S protein;
(b) immunizing animals (e.g., rabbits, mice etc) with the peptides;
(c) collecting blood from the immunized animals; and
(d) testing the antisera collected from animals immunized with the peptides derived from SARS-CoV s protein RBD for neutralizing activity against SARS-CoV.

As an alternative, cells may be immunized in vitro. Spleen cells of an appropriate host may be harvested and contact with the RBD of SARS-CoV S protein. After the immunization, routine procedure for production of monoclonal antibodies may be carried out.

Epitope

This invention further provides the epitope of the RBD of SARS-CoV S protein. In an embodiment, the epitope is determined by the above-described method. In a further embodiment, the epitope is a neutralization epitope.

An isolated polypeptide or recombinant protein containing the sequence of the neutralizing epitope can be used as a vaccine.

The nucleic acid fragment, or the nucleic acid molecule which contains the sequence of a fragment, encoding the sequence of the neutralizing epitope or a vector comprising nucleic acid fragment, or the nucleic acid molecule which contains the sequence of a fragment, encoding the sequence of the neutralizing epitope may be used as vaccine.

This invention also provides a compound containing the sequence or conformation of the epitope. In an embodiment, the compound is a peptide or polypeptide.

This invention further provides a composition comprising a compound which contains the epitope, the isolated peptide or polypeptide.

This invention also provides a vaccine comprising an effective amount of any of the above composition.

This invention provides a method for induction of antibodies against Severe Acute Respiratory Syndrome Virus in a subject comprising administering to the subject the above vaccine.

Increase the Immunogenicity by Linking the IgG Fc Domain

This invention provides a method to increase the immunogenicity of an antigen comprising linking of an IgG Fc domain, its functional fragment or a substance containing the IgG Fc domain or its functional fragment to said antigen.

This invention also provides a composition for increasing the immunogenicity of an antigen comprising an effective amount of

an antigen linked to an IgG Fc domain or its functional fragment or a substance containing an IgG Fc domain or its functional fragment.

In an embodiment of the above method or the composition, the linkage results in a fusion protein.

This invention also provides a method to increase the immunogenicity of an antigen in a subject comprising administering to the subject the antigen linked to the IgG Fc domain or its functional fragment, or a substance containing an IgG Fc domain or its functional fragment.

This invention provides a composition for increasing the immunogenicity of an antigen comprising an effective amount of an antigen and a human IgG Fc domain, its functional fragment, or a substance containing an IgG Fc domain or its functional fragment. The IgG Fc domain can be from rabbit, mouse or any other animals.

The antigen chosen can be of any origin so long as it can illicit immune response. In an embodiment, the antigen encompasses any antigen that can induce antibodies. In a further embodiment, the antigen is derived from an infectious agent. In a still further embodiment, it is of viral origin.

The increase in immunogenicity may result in high level of neutralization antibodies, in high titer of antibodies against the antigen or antibodies with high binding affinity.

Appropriate adjuvant(s) may also be used in the vaccine or the composition to increase the immune response. Said usage of adjuvants is well known in the art. The adjuvants include but are not limited to saponin based adjuvants.

PCT/US2005/019266

This invention further provides the above compositions and a pharmaceutically acceptable carrier, thereby forming pharmaceutical compositions.

This invention also provides a pharmaceutical composition comprising a combination as described above and a pharmaceutically acceptable carrier. For the purposes of this invention, "pharmaceutically acceptable carriers" means any of the standard pharmaceutical carriers. Examples of suitable carriers are well known in the art and may include, but are not limited to, any of the standard pharmaceutical carriers such as a phosphate buffered saline solution and various wetting agents. Other carriers may include additives used in tablets, granules and capsules, etc. Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stearic acid or salts thereof, magnesium or calcium stearate, talc, vegetable fats or oils, gum, glycols or other known excipients. Such carriers may also include flavor and color additives or other ingredients. Compositions comprising such carriers are formulated by well-known conventional methods.

This invention demonstrates that a peptide derived from the HR2 region had inhibitory activity on SARS-CoV infection and it can interact with a peptide derived from HR1 region to form six-helix bundle, resembling the fusion-active core structure of gp41 in HIV. These suggest a model of SARS-CoV entry into target cell: upon binding of RBD on the S1 to ACE2, S2 changes conformation by interaction between the HR1 and HR2 regions to form fusogenic core and bring viral and target cell membrane into close proximity, resulting in virus fusion and entry. This indicates that the fragments containing the functional regions in the S protein may be used as immunogens to induce antibodies to block virus binding or fusion.

PCT/US2005/019266

This invention also provides a method for designing effective and safe subunit vaccines for prevention of SARS using a recombinant fusion protein (designated RBD-Fc, see Fig. 1) containing RBD linked to human IgG Fc fragment (for facilitating BRD purification) as an antigen to immunize rabbits and evaluated the antibody titers of binding RBD and neutralizing SARS-CoV was used in order to design effective and safe subunit vaccines for prevention of SARS.

The invention will be better understood by reference to the Experimental Details which follow, but those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative, and are not meant to limit the invention as described herein, which is defined by the claims which follow thereafter.

Experimental Details

Materials and Methods

Expression of recombinant RBD-Fc and S1-C9 proteins. Plasmid encoding a 193-amino-acid fragment of SARS-CoV S protein, corresponding to the receptor-binding domain, fused with the Fc domain of human IgG1 (RBD-Fc) and plasmid encoding S1 protein (residues 12-672) tagged with C9 at the C-terminus (S1-C9) has been described previously (21, 23). The RBD-Fc and S1-C9 proteins were, respectively, expressed by transfecting 293T cells with the plasmids using Fugene 6 reagents (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's protocol. Supernatants were harvested 72 h post-transfection. Recombinant RBD-Fc fusion proteins were purified by Protein A Sepharose 4 Fast Flow (Amersham Biosciences, Piscataway, NJ), and S1-C9 proteins were purified by affinity chromatography with anti-C9 mouse monoclonal antibody (mAb) 1D4 (National Cell Culture Center, Minneapolis, MN).

Immunization of mice with RBD-Fc. Ten Balb/c mice (4 wks old) were immunized subcutaneously with 10 μg purified RBD-Fc resuspended in PBS (pH 7.2) in the presence of MLP+TDM Adjuvant System (Sigma, Saint Louis, MI) and boosted with the same antigen preparations at 3-wk intervals. Two Balb/c mice as controls were treated in same way as the immunized mice except that RBD-Fc was replaced by PBS. Pre-immune sera were collected before starting the immunization and antisera were collected 4 days after each boost. Sera were kept at 4 °C before use.

Production of rabbit antisera. Rabbit antisera directed against RBD-Fc were produced at Covance Research Products Inc. (Denver, PA) using their standard protocols. Briefly, NZW rabbits were immunized intradermally with 150 μg purified RBD-Fc resuspended in phosphate-buffered solution (PBS, pH 7.2) in the presence of Freund's complete adjuvant (FCA), and boosted with freshly

PCT/US2005/019266

prepared emulsion of the immunogen and Freund's incomplete adjuvant (FIA) at 3-wk intervals. Pre-immune sera were collected before starting the immunization and antisera were collected 10 days after each boost. Sera were kept at 4 °C before use.

Enzyme-linked immunosorbent assay (ELISA). The reactivity of mouse and rabbit sera with various antigens was determined by ELISA. Briefly, 1 μg/ml recombinant proteins (RBD-Fc or S1-C9) or purified human IgG (Zymed, South San Francisco, CA) were used, respectively, to coat 96-well microtiter plates (Corning Costar, Acton, MA) in 0.1 M carbonate buffer (pH 9.6) at 4 °C overnight. After blocking with 2% non-fat milk, serially diluted mouse and rabbit sera, respectively, were added and incubated at 37 °C for 1 h, followed by four washes with PBS containing 0.1% Tween 20. Bound antibodies were detected by addition of HRP-conjugated goat anti-mouse and rabbit IgG (Zymed) , respectively, and the substrate 3,3',5,5'-tetramethylbenzidine (TMB) sequentially. Absorbance at 450 nm was measured by an ELISA plate reader (Tecan US, Research Triangle Park, NC).

Neutralization of SARS-CoV infection. Neutralization of SARS-CoV infection was assessed as previously described (29). Briefly, Vero E6 cells were plated ($5x10^4$ cells/well) in 96-well tissue culture plates and grown overnight. 100 $TCID_{50}$ (50% tissue-culture infectious dose) of SARS-CoV BJ01 strain (Accession number: AY278488) was mixed with an equal volume of diluted mouse and rabbit sera, respectively, and incubated at 37 °C for 1 h. The mixture was added to monolayers of Vero E6 cells. Cytopathic effect (CPE) was recorded on days 3 post-infection as previously described (29). The neutralizing titers represented the dilutions of mouse and rabbit antisera that completely prevented CPE in 50% of the wells (34) as calculated with Reed's method (36).

PCT/US2005/019266

Neutralization of pseudovirus infection. HIV pseudotyped with SARS-CoV S protein (HIV/SARS-CoV S) was prepared as previously described (23, 24). In brief, 293T cells were co-transfected with a plasmid encoding codon-optimized SARS-CoV S protein and a plasmid encoding Env-defective, luciferase-expressing HIV-1 genome (pNL4-3.luc.RE)using Fugene 6 reagents (Boehringer Mannheim). Supernatants containing HIV/SARS-CoV S protein were harvested 48 h post-transfection and used for single-cycle infection of ACE2-transfected 293T cells. Briefly, ACE2-expressed 293T cells were plated (10^4 cells/well) in 96-well tissue-culture plates and grown overnight. The pseudovirus was preincubated with 2-fold serially diluted mouse and rabbit sera, respectively, at 37 °C for 1 h before addition to cells. The culture was re-fed with fresh medium 24 h later and incubated for an additional 48 h. Cells were washed with PBS and lysed using lysis reagent included in a luciferase kit (Promega, Madison, WI). Aliquots of cell lysates were transferred to 96-well Costar flat-bottom luminometer plates (Corning Costar, Corning, NY), followed by addition of luciferase substrate (Promega). Relative light units were determined immediately on the Ultra 384 luminometer (Tecan US).

Inhibition of S1-protein binding to soluble ACE2. Recombinant soluble ACE2 (R&D systems, Inc., Minneapolis, MN) at 2 μg/ml was coated to 96-well ELISA plates (Corning Costar) in 0.1 M carbonate buffer (pH 9.6) at 4 °C overnight. After blocking with 2% non-fat milk, 2 μg/ml S1-C9 was added to the wells in the presence of 2-fold serially diluted mouse and rabbit sera, respectively. After incubation at 37 °C for 1 h, the mAb 1D4 (National Cell Culture Center) was added and incubated at 37 °C for an additional 1 h. After washing, the HRP-conjugated goat anti-mouse IgG (Zymed) and the substrate TMB were used for detection.

PCT/US2005/019266

Measurement of inhibition of S1 binding to cell-expressed ACE2 by flow cytometry. 10^6 stable 293T/ACE2 cells were detached and washed with Hank's balanced salt solution (HBSS) (Sigma, St. Louis, MO). S1-C9 was added to the cells to a final concentration of 1 µg/ml in the presence or absence of rabbit sera at indicated dilutions, followed by incubation at room temperature for 30 min. After thorough washes, the anti-C9 mAb 1D4 was added to the cells to a final concentration of 10 µg/ml and incubated at room temperature for 30 min. Cells were washed with HBSS and incubated with anti-mouse IgG-FITC conjugate (Sigma) at 1:50 dilution at room temperature for an additional 30 min. After washing, cells were fixed with 1% formaldehyde in PBS and analyzed in a Becton FACSCalibur flow cytometer (Mountain View, CA) using CellQuest software.

Experimental Results

Mouse and rabbit antisera directed against RBD-Fc contained high titers of antibodies binding to RBD and S1 domains. Ten mice (M1 to M10) were immunized with RBD-Fc and two control mice (N1 and N2) treated with PBS. Mouse antisera were collected before immunization (pre-immune) and 4 days after each boost at intervals of 3 wks. The serum samples at 1:10,000 dilutions were tested for binding to the recombinant fusion protein RBD-Fc by ELISA. As shown in Figure 2A, the antisera collected 4 days after the first boost showed moderate binding activity. However, this activity significantly increased after the second and third boosts at 3 wks intervals. The antisera collected 4 days after the third boost showed highest RBD-Fc binding activity. Therefore, we used this batch of mouse antisera for the subsequent antibody titration and neutralization experiments. These antisera bound to RBD-Fc in dose-dependent manner with geometric mean titer (GMT) at 1:312,500 (Figure 2B). Since RBD-Fc also contains a human IgG-Fc fragment, the antibodies in the mouse sera may also bind to Fc, in addition to RBD. Therefore,

we tested the binding activity of mouse antisera against the recombinant protein S1-C9, which contains RBD but not Fc. As shown in Figure 2C, mouse antisera bound to S1-C9 in a pattern similar to that shown in the experiments using RBD-Fc as an antigen shown in Figure 2B, although the GMT (1:62,500) of the antibodies against S1-C9 were lower than those to RBD-Fc. This suggests that anti-RBD antibody is one of the major antibody populations in mouse antisera.

Rabbit antisera collected before immunization (pre-immune) and 10 days after each boost at intervals of 3 wks were also tested for their activity of binding to RBD-Fc and S1-C9. As shown in Figure 3A, the antisera (1:10,000) collected 10 days after the 1st boost had the maximum reactivity with RBD-Fc and retained the high levels after the 2nd and 3rd boosts. The GMT of the antisera collected 10 days after the 1st boost was 1:7,812,500 (Figure 3B). Therefore, we used these antiserum samples for the subsequent studies These rabbit antisera also bound to S1-C9 in a dose-dependent manner with GMT of 1:312,500 (Figure 3C), in consistence with the results of using mouse antisera.

Mouse and rabbit antisera against RBD-Fc contained high titers of SARS-CoV-neutralizing antibodies. The antisera were tested for their neutralizing activity using two different assay systems, i.e., infection of SARS-CoV in Vero E6 and of HIV pseudotyped with SARS-CoV S protein (HIV/SARS-CoV S) in 293T cells expressing ACE2. The antisera from 5 mice at 1:10,240 and those from the remaining 5 mice at 1:5,120 fully protected Vero E6 cells from SARS-CoV infection (i.e., no CPE was seen and the cell monolayer remained intact). At higher serum dilutions, the cell number decreased due to the CPE induced by SARS-CoV replication in cells. Here we showed the results obtained from the mouse M8 as an example (Figure 4A). Mean neutralizing antibody titer calculated based on Reed's method (36) was 1:10,862. The pre-immune mouse sera at a 1:40 dilution had no

PCT/US2005/019266

inhibitory activity on SARS-CoV infection. These mouse antisera were also highly effective in inhibiting infection by HIV/SARS-CoV S pseudovirus with 50% neutralizing titer (GMT) of 1:13,636 (Figure 4B), suggesting that the anti-RBD antibodies can inhibit infection by SARS-CoV and pseudovirus containing SARS-CoV S protein. Similarly, both rabbit antisera collected 10 days after the first boost at dilution of 1:10,240 completely inhibited CPE caused by SARS-CoV replication in Vero E6 cells with GMT of neutralization of 1:14,482 (Figure 5). These rabbit antisera also effectively inhibited infection by HIV/SARS-CoV S pseudovirus (Figure 6). The antisera collected 10 days after the second and third boosts possessed comparable neutralizing activity against SARS-CoV and pseudovirus infection (data not shown).

Depletion of anti-Fc antibodies from the antisera directed against RBD-Fc did not affect the RBD-binding and neutralizing activity. Since the recombinant fusion protein RBD-Fc also contains a human IgG Fc fragment, it is expected that this antigen will also induce anti-Fc antibodies. Indeed, the rabbit antisera (1:100) reacted with human IgG-Fc coated in the wells of plates (Figure 7A). However, the anti-Fc antibodies could be depleted from the antisera by passing the antisera through a column conjugated with human IgG since the anti-Fc-depleted antisera had no reaction with human IgG in ELISA (Figure 7A). Anti-Fc-depleted antisera retained the RBD-binding activity (Figure 7B) and neutralizing activity against infection by HIV/SARS-CoV S pseudovirus (Figure 7C), comparable with the untreated rabbit antisera. These results suggest that the anti-Fc antibodies in the antisera induced by human IgG-Fc had no contribution to the RBD-binding and virus-neutralizing activity of the rabbit antisera.

Mouse and rabbit antisera against RBD-Fc effectively blocked RBD binding to ACE2. We tested whether the anti-RBD antibodies in

PCT/US2005/019266

the mouse and rabbit antisera block RBD binding to soluble and cell-associated AEC2 using ELISA and flow cytometry, respectively. Since RBD-Fc can also react with the anti-Fc antibodies in the antisera directed against RBD-Fc, we used S1-C9 which contains only RBD, but not Fc in all the experiments for determining the binding of RBD. In ELISA assay, soluble ACE2 was coated on the wells of ELISA plates and S1-C9 significantly bound to ACE2 (data not shown). Both mouse and rabbit anti-RBD antisera effectively blocked S1 binding to ACE2 in a dose-dependent manner while the pre-immune sera had no inhibitory activity (Figure 8A and 8B). Interestingly, the sera from five mice (M6 to M10) with better activity of inhibiting S1-ACE2 binding had more potent neutralizing activity against SARS-CoV infection in Vero E6 cells than antisera from the mice M1 to M5 (data not shown). Soluble ACE2 coated on plastics may lose the native conformation, so we also used cell expressed ACE2, which is expected to retain the native conformation, for detecting the RBD-binding activity in a flow-cytometric assay. As shown in Figure 8C, S1-C9 significantly bound to ACE2-expressed cells as measured using anti-C9 mAb 1D4 (positive control). If no S1-C9 was added (negative control), only background signals were detected. Rabbit antisera at 1:100 effectively blocked S1 binding to ACE2-expressed cells while pre-immune rabbit sera at the same dilution had no inhibitory activity. The inhibitory activity of the rabbit antisera on S1 binding to ACE2-expressed cells was dose-dependent. Depletion of anti-Fc antibodies from the rabbit antisera did not affect the inhibitory activity of the rabbit antisera on S1-ACE2 interaction (Figure 8D), confirming that the anti-RBD activity is not mediated by anti-Fc antibodies.

Discussion of the results

During the SARS pandemic of 2002/2003, despite the lack of effective and specific therapy, most SARS patients recovered

PCT/US2005/019266

from the acute illness and very few patents could be re-infected by SARS-CoV (http://www.who.int/csr/sars/en). Neutralizing antibodies were detectable in the convalescent sera of SARS patients (37). Passive transmission of the convalescent sera was used for treatment of SARS-CoV infection (http://www.crienglish.com/144/2003-5-7/11@12493.htm). Inoculation of hyperimmune sera from mice infected by SARS-CoV (38) or immunized with MVA/S (35) reduced the titers of SARS-CoV in the respiratory tracts after challenge. Theses data suggest that protective humoral immunity is achievable and vaccines can be developed for prevention of SARS.

A number of vaccine candidates are in clinical trial and preclinical study, including inactivated vaccines, DNA vaccines and vaccinia virus-based vaccines encoding SARS-CoV S protein (9, 10, 34, 35). These agents are effective in inducing protective neutralizing antibody response in animals (34, 35). In the present study, we used a recombinant fusion protein (RBD-Fc) as an immunogen to immunize mice and rabbits since RBD is a key functional domain in the S protein responsible for viral binding to receptor on the target cell (24-26) and contains neutralizing epitopes (39). Antibodies against RBD on the S proteins of other coronaviruses, such as MHV, transmissible gastroenteritis virus (TGEV) and human coronavirus (HCoV-229E)(40-42), and those against receptors for the coronaviruses (43, 44) are highly effective in blocking RBD-receptor interaction and neutralizing infection by the corresponding coronaviruses. We found that the RBD-Fc fusion protein elicited highly potent neutralizing antibody responses in the immunized mice and rabbits and the antisera could completely block SARS-CoV infection at the serum dilutions of 1:5,120 to 1:10,240 (Figures 4 and 5) with geometric mean neutralizing titers of 1:10,381 (mouse antisera) and 1:14,451 (rabbit antisera), about 40-200 fold more potent than those of antisera from mice immunized with DNA vaccines and

PCT/US2005/019266

vaccinia virus-based vaccines encoding the full-length S protein (34, 35).

Since full-length S protein contains RBD and other viral functional domains and multiple neutralizing epitopes, it is expected to induce more potent neutralizing antibodies than RBD alone. One possible reason why RBD actually elicited much higher titers of neutralizing antibodies than full-length S protein is that the latter contains non-neutralizing epitopes that may elicit enhancing antibodies, like those induced by antigenic sites on the envelope glycoproteins of HIV and Ebola virus (45-48). The S proteins from some coronaviruses could also induce enhancing antibodies. For example, immunization of felines with a vaccinia virus vector encoding the S protein of feline infectious peritonitis virus (FIPV) resulted in enhancement of virus replication after virus challenge (49, 50) and the epitopes that elicit enhancing antibodies were localized in the S protein (51). Although no enhanced virus replication was observed in mice immunized with DNA vaccines and vaccinia virus-based vaccines encoding SARS-CoV S protein (34, 35), this may not exclude the possibility that the enhancing antibody titers are lower than neutralizing antibody titers. In such case, enhancing antibodies may suppress or "neutralize" the neutralizing-antibody activity, resulting in reduced neutralizing-antibody titers.

Another possibility of the recombinant fusion protein RBD-Fc being able to induce highly potent neutralizing antibodies may be because the antigen contains human IgG Fc fragment. Antigen-presenting cells (APCs), such as dendritic cells and monocytes/macrophages, can capture, process and present antigens to T helper cells, which regulate antibody production. It has been shown that APCs express the high-affinity receptor for IgG Fc, FcgammaRI (also named CD64) and low-affinity receptor, FcgammaRIII (CD16) (52, 53). Through these receptors, APCs can

PCT/US2005/019266

accelerate presentation of the immune complex containing antigen and antibody IgG and enhance antibody response against the immune complex, resulting in autoimmune diseases (53). However, if the antigen is a viral protein, such as RBD in the SARS-CoV S protein, conjugation of human IgG Fc to it may accelerate presentation of RBD to immune cells for eliciting highly potent anti-RBD antibody response and neutralizing SARS-CoV infection.

The mouse and rabbit antisera directed against RBD are effective in binding to RBD on the S1 domain of SARS-CoV S protein (Figures 2 and 3) and blocking RBD binding to soluble and cell-expressed ACE2 (Figure 8). These confirm that the mouse and rabbit antisera contain antibodies specifically targeted to RBD. Although we have not tested the protective activity of the mouse and rabbit anti-RBD antibodies in animal models against SARS-CoV challenge, the high neutralizing titers of these antisera tested in vitro suggest that RBD-Fc may induce strong protective immunity in animals and humans, considering that the effective protection against SARS-CoV infection can be achieved by the convalescent sera from SARS patients with neutralizing antibody titers ranging from 1:20 to 1:1,280 (37) and by antisera from mice immunized by DNA vaccines and vaccinia virus-based vaccines encoding S protein with low neutralizing antibody titers (1:50 to 1:284) (34, 35).

The sequence of S proteins, especially the S1 domains, of most coronaviruses are highly variable (14), which is a major concern in developing effective vaccines against virus strains with distinct genotypes and phenotypes. However, recent studies have shown that SARS-CoV strains are quite stable and do not change as much as that was originally predicted (10). At the early phase of SARS endemic in 2002/2003, 5 out of the 193 amino acid residues in the RBD of SARS-CoV S protein are variable due to the positive selection pressure in the process of transition from animal (e.g., palm civet) SARS-like-CoV to human SARS-CoV.

PCT/US2005/019266

However, at the middle and late phases (most virus strains were isolated from SARS patients during these two phases), there is no mutation in the RBD sequence.(6). Furthermore, the conformation of RBD is relatively conserved to ensure the binding of virus with different subtypes to a specific receptor on the target cells, even though the linear sequence of RBD may be variable. One example is B12 mAb which recognizes the neutralizing epitopes on the CD4-binding domain on HIV-1 gp120 and neutralizes a broad range of HIV-1 primary isolates, although the linear sequences of CD4-binding regions in gp120 from the corresponding strains are highly variable (54, 55). Our data have shown that antibodies in the mouse and rabbit antisera directed against RBD may primarily recognize conformational epitopes on RBD since the antisera did not react with any of the peptides overlapping the RBD sequence (He et al., unpublished data). These suggest that anti-RBD antibodies may have neutralizing activity with specificity against a broad spectrum of SARS-CoV strains.

In summary, the recombinant fusion protein RBD-Fc is an ideal vaccine candidate since it induces highly potent antibodies to block S1-receptor interaction and to neutralize SARS-CoV infection and has low level of risk compared with inactivated viruses or live attenuated virus vectors. Therefore RBD-Fc can be further developed as an effective and safe subunit vaccine for prevention of SARS.

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What is claimed is:

1. A vaccine comprising an effective amount of isolated polypeptide or recombinant protein containing the sequence of receptor-binding domain in the Severe Acute Respiratory Syndrome associated coronavirus spike protein or a functional fragment thereof.

2. The vaccine of claim 1 wherein the receptor-binding domain comprises a full or partial sequence containing
NITNLCPFGEVFNATKFPSVYAWERKKISNCVADYSVLYNSTFFS
TFKCYGVSATKLNDLCFSNVYADSFVVKGDDVRQIAPGQTGVIA
DYNYKLPDDFMGCVLAWNTRNIDATSTGNYNYKYRYLRHGKLRPFERDIS
NVPFSPDGKPCTPPALNCYWPLNDYGFYTTTGIGYQPYRVVVLSFELLNAP
ATV. (SEQ ID NO: 1)

3. A vaccine comprising an effective amount of a nucleic acid molecule comprising the sequence of a fragment which encodes the sequence of receptor-binding domain in the Severe Acute Respiratory Syndrome associated coronavirus spike protein or a functional fragment thereof.

4. An isolated polypeptide other than S1 protein of the Severe Acute Respiratory Syndrome Virus, comprising sequence of receptor-binding domain in the Severe Acute Respiratory Syndrome associated coronavirus spike protein or a functional fragment thereof.

5. The isolated polypeptide of claim 4, comprising full or partial sequence containing
NITNLCPFGEVFNATKFPSVYAWERKKISNCVADYSVLYNSTFFS
TFKCYGVSATKLNDLCFSNVYADSFVVKGDDVRQIAPGQTGVIA
DYNYKLPDDFMGCVLAWNTRNIDATSTGNYNYKYRYLRHGKLRPFERDIS
NVPFSPDGKPCTPPALNCYWPLNDYGFYTTTGIGYQPYRVVVLSFELLNAP
ATV. (SEQ ID NO: 1)

6. The isolated polypeptide of claim 4 or 5, linked to a substance comprising an IgG Fc domain, its functional fragment or a substance containing an IgG Fc domain or its functional fragment.

7. The linked polypeptide of claim 6 comprising a full or partial sequence containing THTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPQVKFNWYVDGV QVHNAKTKPREQQYNSTYRVVSVLTVLHQNWLDGKEYKCKVSNKALPAPIEKTISKAKGQ PREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDG SFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG. (SEQ ID NO: 2)

8. The isolated linked polypeptide of claim 6 or 7, wherein the IgG Fc is from human, rabbit, mouse and other animals.

9. The isolated linked polypeptide of claim 6 or 7 which is a fusion protein.

10. A nucleic acid molecule comprising the sequence of a fragment which encodes the polypeptide of any of claims 4-9.

11. A vector comprising a nucleic acid molecule comprising the sequence of a fragment of claim 10.

12. A composition comprising an effective amount of the isolated polypeptide of any of claims 4-9, the nucleic fragment of claim 10, or the vector of claim 11.

13. A pharmaceutical composition comprising the isolated polypeptide of any of claims 4-9 or the nucleic acid molecule comprising the sequence of a fragment of claim 10 or the vector of claim 11 and a pharmaceutically acceptable carrier.

14. A method for induction of antibodies against Severe Acute Respiratory Syndrome Virus in a subject comprising administering to the subject the vaccine of claim 1 or 2 or an effective amount of the isolated polypeptide of any of claim 4-9 or the nucleic acid molecule comprising the sequence of a fragment of claim 10 or the vector of claim 11 or the composition of claim 11 or 12.

15. The method of claim 14, wherein the antibodies are neutralizing.

16. The method of claim 14 or 15, wherein the subject is a human or animal.

17. The method of any of claims 13-16, wherein the antibody is polyclonal antibody.

18. The method of any of claim 13-16, wherein the antibody is monoclonal antibody.

19. An antibody generated by the method of any of claim 13-18.

20. An anti-idiotypic antibody or a functional portion thereof, against the monoclonal antibody of claim 18.

21. A vaccine comprising an effective amount of the anti-idiotypic antibody of claim 20 or a functional portion thereof.

22. A method for determining the neutralizing epitope contained in S1 of the Severe Acute Respiratory Syndrome Virus comprising steps of:

(a) generating peptide from the RBD sequence of SARS CoV S protein;

(b) immunizing animals with the peptides;

(c) collecting blood from the immunized animals; and

(d) testing the antisera collected from animals immunized with the peptides derived from SARS-CoV S protein RBD for neutralizing activity against SARS-CoV.

23. The method of claim 22, wherein the animals are rabbits or mice.

24. The determined epitope by the method of claim 22, or an isolated peptide containing the sequence or conformation of the said epitope or a nucleic acid molecule comprising the sequence of a fragment encoding said epitope.

25. A compound containing the epitope of claim 23 or its functional equivalent.

26. A composition comprising the epitope of claim 23, the isolated peptide of claim 24 or the compound of claim 25 or a nucleic acid molecule comprising the sequence of a fragment encoding said epitope.

27. A vaccine comprising an effective amount of composition of claim 26.

28. A method for induction of antibodies against Severe Acute Respiratory Syndrome Virus in a subject comprising administering to the subject the vaccine of claim 27.

29. A method to increase the immunogenicity of an antigen comprising linking of an IgG Fc domain, its functional fragment or a substance containing the IgG Fc domain or its functional fragment to said antigen.

30. A composition for increasing the immunogenicity of an antigen comprising an effective amount of an antigen linked to an IgG Fc domain or its functional fragment or a substance containing an IgG Fc domain or its functional fragment.

31. The method of claim 29 or the composition of claim 29, wherein the antigen is linked to the IgG Fc domain, or its functional fragment to form a fusion protein.

32. A method to increase the immunogenicity of an antigen in a subject comprising administering to the subject the antigen linked to the IgG Fc domain or its functional fragment, or a substance containing an IgG Fc domain or its functional fragment.

33. A composition for increasing the immunogenicity of an antigen comprising an effective amount of an antigen and an IgG Fc domain, its functional fragment, or a substance containing an IgG Fc domain or its functional fragment.

34. The method of any of claims 29-32 or the composition of claim 33, wherein the antigen is derived from an infectious agent.

35. The method of claim 34, wherein the increase in immunogenicity results in high level of neutralization antibodies.

36. The method of any of claims 29-32 or the composition of claim 33, wherein the antigen is any antigen that can induce antibodies.

37. The method of claim 36, wherein the increase in immunogenicity results in high titer of antibodies against the antigen.

38. The method of claim 36, wherein the increase in immunogenicity results in antibodies with high binding affinity.