HUMAN MONOCLONAL ANTIBODY WITH SPECIFICITY FOR DENGUE VIRUS SEROTYPE 1 E PROTEIN AND USES THEREOF

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to U.S. Provisional Patent Application No. 61/423,085, filed December 14, 2010, the disclosure of which is hereby incorporate by reference in its entirety for all purposes.

SEQUENCE LISTING

[0001.1] The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety.

FIELD

[0002] The invention relates to human neutralizing monoclonal antibodies to Dengue virus, in particular, serotype 1. The invention further relates to compositions and methods for the treatment or prevention of infection by Dengue virus in a vertebrate subject. Methods are provided for administering antibodies to the vertebrate subject in an amount effective to reduce, eliminate, or prevent relapse from infection.

BACKGROUND

[0003] Dengue is the most significant mosquito-borne viral disease affecting humans. At present close to 2.5 billion people living in more than 100 dengue endemic countries in the tropical/sub-tropical belt are considered to be at risk of dengue infection. The urban dwelling mosquito species, *Aedes aegypti* is the principal transmitter of the virus to humans. Infection with dengue virus can result in a spectrum of clinical manifestations ranging from asymptomatic infection through dengue fever (DF), an acute febrile disease, to dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) which are severe, life-threatening complications typified by vascular leakage. Current treatment is limited to the use of analgesics to alleviate the symptoms and there are no vaccines available. Dengue diseases affect 50 million people yearly, with frequent and recurrent epidemics. The 1990's saw a return of dengue diseases in various areas of the world despite stringent mosquito controls, peaking with the largest ever outbreak in 2005 in Singapore. Over 80% of the reported cases were young adults with an associated impact on their ability to work plus significant healthcare costs for their treatment. Hence, alternatives to dengue vaccines, such as passive antibody therapies and/or antivirals are needed urgently to help control dengue associated diseases in the immediate term. These proposed therapeutics have the potential to help large numbers of infected individuals even if only applied to individuals at risk of developing the severe forms of disease (around 10% of the total). With the increasing prevalence of dengue

in developed nations such as the Southern United States plus Australia, and the absence of a vaccine, such an antibody would provide a useful medication. The present invention provides fully human monoclonal antibodies to satisfy these and other needs.

SUMMARY

[0004] Described herein are compositions and methods for the treatment or prevention of Dengue virus infection in a vertebrate subject.

[0005] In particular, disclosed herein is an example of the generation of fully human neutralizing monoclonal antibodies from patients newly recovered from infection with Dengue serotype 1. The antibody exhibits both prophylactic and therapeutic activity in blocking Dengue serotype 1 infection *in vitro* and *in vivo* and can form the basis of a new medication. The invention utilizes a method for preparing immortalized memory B cells from convalescent patients by purifying their CD22 positive cells from a blood sample taken 60 days after the patient has recovered from infection. The purified B cells are then immortalized, by employing Epstein Barr Virus (EBV) infection. This method generates a panel of immortalized memory B cell lines capable of producing fully human antibodies which can be screened for specificity for Dengue virus. These B cell lines can then be used as an enriched source of immunoglobulin templates for the identification and cloning of recombinant monoclonal antibodies with neutralizing activity for Dengue virus *in vitro* and *in vivo*. As disclosed herein, we describe the isolation, screening, cloning and *in vitro/in vivo* characterization of the first fully human monoclonal antibody specific for Dengue Virus serotype 1.

[0006] In one aspect, the present invention provides an isolated antibody or fragment thereof that binds to a Dengue virus serotype 1 envelope protein or fragment thereof, wherein the antibody is a human antibody with neutralizing activity.

[0007] In various embodiments of this aspect, the antibody or fragment thereof can be (a) a whole immunoglobulin molecule; (b) an scFv; (c) a Fab fragment; (d) an F(ab')2; or (e) a disulfide linked Fv.

[0008] In other embodiments, the antibody or fragment thereof can comprise a heavy chain immunoglobulin constant domain selected from the group consisting of: (a) a human IgM constant domain; (b) a human IgG1 constant domain; (c) a human IgG2 constant domain; (d) a human IgG3 constant domain; (e) a human IgG4 constant domain; or (f) a human IgA1/2 constant domain.

[0009] In yet other embodiments, the antibody or fragment thereof can comprise a light chain immunoglobulin constant domain which can be: (a) a human Ig kappa constant domain; or (b) a human Ig lambda constant domain.

[0010] In additional embodiments, the antibody or fragment thereof comprises a heavy chain comprising at least one CDR selected from the group of CDR sequences shown in Figure 4(B).

[0011] In further embodiments, the antibody or fragment thereof comprises a light chain comprising at least one CDR selected from the group of CDR sequences shown in Figure 4(B).

[0012] In other embodiments, the antibody or fragment thereof comprises a heavy chain comprising three CDR sequences as shown in Figure 4(B).

[0013] In other embodiments, the antibody or fragment thereof comprises a light chain comprising three CDR sequences as shown in Figure 4(B).

[0014] In further embodiments, the antibody or fragment thereof comprises a heavy chain framework of IGHV1-2*02 and at least one of the CDR sequences as shown in Figure 4(B).

[0015] In yet further embodiments, the antibody or fragment thereof comprises a light chain framework of IGKV3-20*01 and at least one of the CDR sequences as shown in Figure 4(B).

[0016] In one embodiment, the antibody comprises the heavy chain sequence shown in Figure 4(B).

[0017] In another embodiment, the antibody comprises the light chain sequence shown in Figure 4(B).

[0018] In yet another embodiment, the antibody is 14c10, clone 8.

[0019] In some embodiments, the antibody or fragment thereof binds to an antigen with an affinity constant (K_D) of less than 1×10^{-8} M.

[0020] In other embodiments, the antibody or fragment thereof binds to an antigen with an affinity constant (K_D) of less than 1×10^{-9} M.

[0021] In further embodiments, the antibody or fragment thereof is derived from a B cell of a patient who has recovered from Dengue virus infection.

[0022] In further embodiments, the antibody or fragment thereof binds across two envelope proteins in a virus. In some embodiments, the binding across two envelope proteins comprises binding to DI and the hinge between DI and II on one E protein and DIII of a neighboring E protein.

[0023] In another aspect, the present invention provides an antibody or fragment thereof that binds to a Dengue virus having the binding specificity of 14c10, clone 8.

[0024] In a further aspect, the present invention provides a pharmaceutical composition comprising the antibody or fragment thereof according to any one of the relevant aspects and embodiments above and a pharmaceutically acceptable carrier effective to reduce or prevent Dengue virus infection in a subject. In some embodiments, the pharmaceutical composition can further comprise a second agent, for example, an antiviral drug or an analgesic drug.

[0025] In a further aspect, the present invention provides a method of passive immunization comprising administration to a subject an effective amount of the antibody or fragment thereof according to any one of the relevant aspects and embodiments above.

[0026] In an additional aspect, the present invention provides a method of treatment of Dengue virus infection comprising administration to a subject in need thereof an amount of antibody or fragment thereof according to any one of the relevant aspects and embodiments above, effective to reduce or prevent the disease.

[0027] In some embodiments, the antibody is administered intravenously (IV), subcutaneously (SC), intramuscularly (IM), transdermally, or orally.

[0028] In other embodiments, the antibody is administered in an amount in the range of 1 to 100 milligrams per kilogram of the subject's body weight.

[0029] Such administration can further comprise administration of a second agent, which can, for example, be an antiviral drug or an analgesic drug.

[0030] In another aspect, the present invention provides a method of generating a neutralizing antibody against a Dengue virus by: (a) identifying an individual who has recently recovered from Dengue virus infection; (b) obtaining B-cells from the individual; (c) immortalizing the B-cells from (b); and (d) assaying the immortalized B-cells from (c) for Dengue virus neutralization.

[0031] In embodiments of this aspect, the B cells are CD 22+. In further embodiments, the B cells are immortalized with EBV.

[0032] In other aspects, the present invention provides an isolated nucleic acid encoding the antibody or fragment thereof according to any one of the relevant aspects and embodiments above. Such isolated nucleic acids can be contained in an expression vector. Such expression vectors can be contained within a host cell, such as a bacterial, eukaryotic, or mammalian cell.

BRIEF DESCRIPTION OF THE DRAWINGS

[0033] Figure 1: Flowchart of the process of antibody screening, expression and characterization. CD22+ B cells from Dengue infected patients admitted to National University Hospital (NUH) were isolated. These B cells were immortalized with EBV in the presence of a polyclonal B cell activator (2.5μg/ml CpG sequences, IL2 and IL4) which were added to enhance the efficiency of immortalization. B cells were plated at 30cells/well in 96 wells round bottom wells with $1x10^5$ allogenic, irradiated PBMCs obtained from buffy coats. After two weeks, supernatants from these clones were screened by ELISA, PRNT and CPE for binding/neutralizing activity. mRNA of the positive B cell lines was extracted and the heavy and light chains sequences of the antibody cloned into an in-house pCMV vector and tranfected into Freestyle® 293F cells to produce high concentrations of recombinant antibodies. The recombinant antibodies with the desired specificity were identified and further characterized.

[0034] Figure 2: Screening of supernatants from immortalized B cell lines with CPE and PRNT for Dengue neutralizing activity. (A) BHK-21 cells were challenged with DV in the presence of supernatants derived from EBV immortalized B-cell lines. (2000 cell lines per patient were screened using this approach). The cytopathic effect was assessed by staining the remaining intact cells with crystal violet elution with acetic acid and determination of the absorbance at 595 nm. The assay endpoint was defined as a 50% cytopathic effect and the viral concentration was optimized. Test supernatants were screened initially at a dilution of 1 in 4. Top 10% of clones were re-tested by PRNT. **(B)** Generation of human B lymphocyte cell line secreting neutralising human antibodies against Dengue. BHK cells at 80% confluency were infected with Dengue virus for 3 days. Viral plaques were visualised using crystal violet dye (Sigma- Aldrich, Singapore) that binds to viable cells. Supernatants from B cell clones (derived from a convalescent Dengue 1 infected individual) were tested for neutralising activity. Dengue 1 (50 pfu) was incubated with cell culture supernatants (diluted ¼) for 1 hour prior to addition to BHK cells. Cell line 14c10 was found to secrete antibodies that significantly reduced plaque numbers.

[0035] Figure 3: Antibody templates expressed by B cell line 14C10 and the associated CDR amino acid sequences. (A) Plasmid map demonstrating restriction enzyme sites and cloning heavy and light chains inserts for generation of a recombinant human IgG1 antibody using identified templates from 14c10. **(B)** All the identified and cloned Heavy and light chain sequences of 14c10 with their CDR regions (CDR 1, CDR 2 and CDR 3

respectively) plus 12 permutations of Heavy and Light Chain combinations to make different recombinant antibodies.

[0036] Figure 4: Antibody template 14c10.8 encodes a recombinant antibody with binding activity for dengue serotype 1. **(A)** Sandwiched ELISA employed to test all recombinantly expressed antibodies derived from the B cell line 14c10 expressed and purified from supernatants of 293F. Template number 8 clearly gives a positive signal for dengue virus serotype 1. **(B)** Full nucleotide and amino acid sequence of 14c10.8 Heavy and Light Chains with CDR regions highlighted.

[0037] Figure 5: Serotype specificity of recombinant 14c10.8 antibody with PRNT and ELISA. **(A)** Sandwiched ELISA showing specificity of recombinant IgG1 14c10 antibody against live whole dengue virus serotype 1. No observable binding activity for Dengue serotypes 2,3 or 4. **(B)** PRNT data showing specificity of recombinant 14c10.8 antibody against Westpac 74 Dengue virus serotype 1. No significant neutralizing activity was detected for Dengue serotype 2,3 or 4. **(C)** Raw data of PRNT showing serotype specificity of 14c10.8 for Dengue virus serotype 1.

[0038] Figure 6: 14c10.8 exhibits homotypic antibody dependent enhancement (ADE) but no heterotypic antibody dependent enhancement for in vitro dengue infection. Serially diluted 14c10.8 antibody was incubated with an equal volume of virus (MOI of 1) for 1hr at 37°C then transferred to the human myelomonocytic cell line K562 (the cell line usually employed for ADE assays) and incubated at 37°C for 4 days. Supernatants were then harvested from the infected K562 cells and the resulting viral titre assessed by PRNT. ADE is defined as increased viral titres compared to controls where no antibody is added (dotted blue line). Data demonstrated presence of ADE in dengue virus serotype 1 but not in serotype 2, 3 and 4. This observation suggests that 14c10.8 should be a safe antibody to give to dengue 1 infected patients provided that it's given at neutralizing rather than enhancing concentrations.

[0039] Figure 7: Conversion of 14c10.8 to different human IgG sub-classes has an impact on its homotypic enhancement activity. We converted 14c10 from a human IgG1 to a human IgG3 and human IgG4 using the constructs outlined. These were expressed as recombinant antibodies in 293F cells then purified on Protein-A sepharose columns for further testing. We testing for homotypic enhancement using the K562 cell lines as described in Fig 6. IgG3 exhibits maximal enhancing activity whilst IgG1 is intermediate and IgG4 has the lowest levels of enhancing activity.

[0040] Figure 8: 14c10.8 is specific for Dengue virus E-protein. **(i)** Cells were infected with DV for two days. Upon which, cells were lysed and S^{32} methionine was added to the mixture of the virus to incorporate the radioactive compound. Antibody was added to the mixture followed by the addition of Protein A-agarose beads were then added and incubated for 1 hr at 4°C. After washing, proteins were eluted with non-reducing loading buffer and run on a 15% SDS–polyacrylamide gel followed by silver staining according to the manufacture protocol (SilverQuest staining kit, Invitrogen). A 56 Kd band corresponds to E protein of Dengue virus. **(ii)** Purified whole dengue virus (denatured and non-denatured) was loaded onto non-denaturing gel and transferred to a membrane to be blotted with 14C10 antibody. Results showed that 14C10 has weak binding to a linear epitope on dengue E protein.

[0041] Figure 9: Neutralization activity of recombinant 14c10 antibody against various dengue serotype 1 genotypes. Increasing concentrations of antibody were added to 50 plaque-forming units (p.f.u.) of various genotypes of Dengue virus serotype 1 (viral genotype name is provided in parenthesis) and incubated at 37 °C for 1 hr. 100µl of mixture was added to a monolayer of BHK-21 cells in a 24 well plate and incubated for 1hr at 37 °C. Supernatant was removed and 1ml of 2% (w/v) carboxyl methyl cellulose in RPMI plus 2% FBS was layered onto the infected cells. After further incubation at 37 °C for 4 days, the wells were stained with 0.5% (w/v) crystal violet dissolved in 25% (v/v) formaldehyde to visualize the plaques.

[0042] Figure 10: 14c10 exhibits both prophylactic and therapeutic activity *in vivo*: **(A)** Prophylactic activity of 14c10.8 was observed by injecting AG129 (n=6) mice with various concentrations of antibody 24hours prior to infection with dengue serotype 1. A single therapeutic dose of 250 µg/mouse of antibody was given to a single cohort (n=6) 24hours after dengue virus infection. The resulting viremia was quantified in the blood serum of infected mice by PRNT 4 days post infection. **(B)** 14c10.8 exhibits prophylactic activity at concentrations of 1-5 µg/mouse. At lower concentrations of antibody there is some evidence of enhanced infection.

[0043] Figure 11: HM14c10 is a human antibody specific for DENV1. (A) HM14c10 exhibits neutralization activity specific for DENV1 with 50% and 90% PRNT values of 0.328 µg/ml and 1.313 µg/ml, respectively. (**B**) HM14c10 induces homotypic ADE for DENV1 at sub-neutralizing concentrations but no heterotypic ADE for DENV2, DENV3 or DENV4. HM4G2 induces ADE activity for all 4 serotypes (**C**) (**a**) The Fab fragment or mutation (N297Q) of the IgG1 Fc region of HM14c10 significantly reduced homotypic ADE. (**b**)

Different subclasses of human IgG (HM14c10) mediate differential levels of homotypic ADE. (**D**) HM14c10 is highly neutralizing to multiple DENV1 genotypes compared to HM4G2. The genotypes are indicated in brackets beside the virus designation. Error bars represent standard deviations of triplicate samples, and all experiments were conducted at least three times.

[0044] Figure 12: HM14c10 binds a virus quaternary structure-dependent epitope.. (**A**) CryoEM map of Fab 14c10:DENV1 complex showing 120 Fabs (blue) binding to 180 E proteins on virus surface (cyan). Black triangle represents an asymmetric unit. (**B**) View of connecting densities of Fab HM14c10(I) to E protein epitope (purple spheres). E protein E-DI, E-DII and E-DIII are colored in red, yellow and blue, respectively. (**C**) Densities of Fab molecules on E protein Cα chains in two asymmetric units. Fab HM14c10(I) and HM14c10(II) are the two independent molecules in an asymmetric unit. (**D**) Epitopes of Fab HM14c10(I) (purple spheres) and HM14c10(II) (cyan spheres) on the three E proteins (shaded in grey) in an asymmetric unit.

[0045] Figure 13: HM14c10 blocks DENV1 attachment to BHK cells and exhibits potent protective activity *in vivo*. (**A**) Time lapse confocal microscopy demonstrating DENV1 infection of BHK host cells in the presence of (**a**) Isotype control mAb, (**b**) HM4G2 and (**c**) HM14c10 mAb. Left panels: DENV1 and Mabs were labeled with Alexafluor-647 (red) and Alexafluor-488 (green), respectively. Right panels showing cell boundaries (white dotted lines) and the distribution of DENV1 in cells. (**B**) Close-up of live infection events. DENV1 are observed inside BHK cells from 18 min in the isotype controls and from 28 min with HM4G2. HM14c10:DENV1 complexes are unable to attach to BHK cells. (**C**) Internal red fluorescence intensity of 120 randomly selected cells quantified as a measure of virus internalization over 1 h. 1-way ANOVA utilized for comparison of 3 groups. **$p<0.0001$. (**D**) HM14c10 is tested for use as a prophylaxis and therapeutic agent; antibody is administered to DENV1 infected AG129 mice at day 0 and day 2 post-infection, respectively. HM14c10 showed protective response whether the virus is injected (**a**) sub-cutaneously or (**b**) intraperitoneally. Level of blood viremia is assayed at day 3 or 4 respectively post infection by plaque assay. N=5 in both models and T-test employed for comparison of sample sets, ** $p<0.0001$, * $p<0.05$ compared with PBS controls.

[0046] Figure 14: Identification and recombinant expression of a fully human antibody with neutralizing activity for Dengue Virus. (**A**)(**a**) Two thousand EBV-B-cell lines were generated from a DENV1 infected patient and supernatants screened by ELISA for

binding activity to DENV1 but not DENV2, 3 or 4. Seven positive EBV-BCL cell lines were identified. (**b**) A plaque reduction neutralization test (PRNT) was carried out to test for neutralizing activity. The data is expressed as a PRNT100 (i.e. complete neutralization) at the highest dilution factor and is the average value from 3 experiments. (**B**)(**a**) Schematic of pTT5 vector utilized to express antibody Heavy Chain and Light Chain templates derived from EBV-BCL in HEK293 cells. (**b**) Twelve recombinant human IgG1 mAbs were cloned and expressed from the EBV-BCL 14c10 cell line and tested for binding activity to DENV1 by ELISA. A humanized mouse monoclonal 4G2 antibody (HM4G2) was employed as a positive control. Recombinant antibody template number 8 (termed HM14c10) exhibited binding activity for DENV1. (**C**)(**a**) PRNT activity of HM14c10 on DENV1, 2, 3 and 4. (**b**) HM14c10 was tested for binding activity to DENV1, 2, 3 and 4 by ELISA. These data represent the mean of 3 experiments and error bars equal standard deviation from the mean of triplicate sample sets.

[0047] Figure 15: HM14c10 exhibits binding activity for multiple DENV1 clinical isolates. HM14c10 binding activity for several DENV1 isolates was compared to a humanized mouse monoclonal antibody HM4G2 at various concentrations using an established ELISA protocol. All DENV1 isolates were employed at $1x10^6$ pfu/ml and coated overnight at 4 °C with HB112 used as a capture reagent. HM14c10 or HM4G2 antibody were added at 5 μg/ml and anti-human IgG HRP conjugates were utilized for detection of binding activity.

[0048] Figure 16: Fit of the post-fusion crystal structure of DENV1 E proteins into the cryoEM map of Fab HM14c10 complexed with Dengue 1 virus. (**A**) Top view of the fitted dengue 1 E proteins. The cryoEM map is displayed at a high contour level of 5.5σ so that clear outline of E protein densities can be observed. At this contour level, the Fab densities disappeared, indicating that not all available E protein epitopes are occupied by Fab molecule on the virus surface. The electron densities of the virus surface were interpreted by fitting in the crystal structure of the post-fusion structure of DENV1 E protein (*18*). Since the crystal structure of the DENV1 post-fusion E protein does not fit well into the cryoEM map as a rigid body, the three domains of the E protein had to be fitted separately. Domains I, II and III of the E protein are colored in red, yellow and blue, respectively. E proteins from two asymmetric units are shown here with one asymmetric unit indicated with a triangle. (**B**) Side view of the fitted E proteins on the surface of DENV1. Densities of the Fab molecules, E protein ectodomain and transmembrane (Tm) helices can be observed. Densities corresponding to glycans at position Asn159 on two adjacent E proteins are marked with

arrowheads and the position of outer and inner leaflet of the lipid bilayer are indicated. The cryoEM map is shown at 2.5σ contour level.

[0049] Figure 17: Stereo-diagram of the Fab HM14c10 and E protein binding interface. Density of Fab HM14c10(II) shows clear connections to E proteins on the virus surface. Contact residues are indicated with spheres. CryoEM density is shown at 2.5σ contour level.

[0050] Figure 18: Superposition of the variable regions of homology model of HM14c10 (green) with reference human monoclonal antibody (PDB code 2GHW) (blue). Figure is showing (**A**) side and (**B**) top view of the antibody variable regions.

[0051] Figure 19: Fitting of the homology model of HM14c10 variable region into HM14c10:DENV1 cryoEM density map. (A) The densities corresponding to the individual chains (a and b) of the antibody variable region are circled from the cryoEM map. The contact residues of the fitted E protein are indicated with cyan spheres. E-DI, E-DII and E-DIII are colored in red, yellow and blue, respectively. **(B)** The homology model light and heavy chains were fitted separately into the variable region of the Fab cryoEM densities. [a] For designation of Fab position see Fig. 12. [b] For designation of Fab density see (A). [c] The fits of the homology model into the HM14c10:DENV1 cryoEM map (set at a contour level of 3σ) were optimized by using the fit-in-map function in Chimera (*35*). **(C)** The fitted HM14c10 variable region homology model (green) showing the CDRs in magenta. The fit shown has light chain in Fab density a, and heavy chain in Fab density b.

[0052] Figure 20: HM14c10 epitope on dengue serotype 1 (genotype PVP159) and comparison of the epitope with (A) other DENV1 genotypes and (B) dengue serotypes and West Nile virus (WNV). Common amino acid residues between the epitopes recognized by Fab HM14c10(I) and Fab HM14c10(II) in an asymmetric unit are colored in green. Residues that are uniquely recognized by Fab HM14c10(I) or Fab HM14c10(II) are colored in purple and cyan, respectively. The amino acid sequences of the epitopes recognized by Fab HM14c10 are conserved within DENV1 genotypes, but not across dengue serotypes or West Nile virus. This is consistent with the observation that Fab HM14c10 binds to most dengue 1 genotypes, but does not cross-react with other dengue serotypes or flaviviruses with shaded antibody footprints (**a**) at position X1 and II or (**b**) position X2 and I.

[0053] Figure 21: Infectivity and *in vivo* efficacy of labeled DENV1. (**A**) Live DENV labelling was conducted as previously described (*22*). The infectivity and viability of labeled virus was tested by plaque assay through titration on BHK cells. (**B**) The in vivo efficacy of

HM14c10 was tested in two *in vivo* models employing different strains/concentrations of DENV1 virus plus different modes of viral delivery. A schematic of both of these models is shown. (**a**) In model 1, $1x10^6$ pfu of EHID1 strain is injected sub-cutaneously (S.C.) and the serum viremia monitored by plaque assay 4 days later. Prophylaxes are given 24 h before DENV1 infection and therapeutic applications at day plus 2 post infection. (**b**) A second more aggressive DENV1 infection model was also employed. Mice were injected intraperitoneally with $1.25x10^7$ pfu of the Westpac strain of DENV1. Virus infection plus prophylactic and therapeutic treatments were administered via intraperitoneal (I.P.) injection at the same time points as model 1. In this model plasma viremia peaks at day +3 post infection and this is where the effects of the administered antibody on serum viremia is measured. Controls in both groups were given an equal volume of sterile saline.

[0054] Figure 22: Comparison of epitope bound by a West Nile virus antibody CR4354 and dengue 1 specific HM14c10. (**A**) Fit of HM CR4354 and HM 14c10 to E proteins on WNV (left) (*25*) and DENV (right), respectively. CryoEM density is displayed at 2.8σ (CR4354:WNV) or 2.5σ (HM14c10:DENV1) contour level. (**B**) An asymmetric unit of WNV (left) and DENV1 (right) with antibody CR4354 or HM14c10 footprints shown in spheres. Epitopes at the two independent binding sites in an asymmetric unit are colored in purple and cyan. The three E proteins in an asymmetric unit are shaded in gray. An asymmetric is shown as black triangle. (**C**) Comparison of residues in the two independent epitopes (**a** and **b**) between CR4354 (on WNV) and HM 14c10 (on DENV). Residues in the two independent epitopes are colored as in (**B**).

DETAILED DESCRIPTION

[0055] The present invention generally relates to compositions and methods for the prevention or treatment of Dengue virus infection in a vertebrate subject. In particular, we have isolated CD22+ B cells from Dengue infected patients admitted to the Infectious disease division of National University Hospital (NUH). These B cells were immortalized as polyclonal cell lines with EBV *in vitro*. The polyclonal B cell activator (CpG sequences) was added to enhance the efficiency of B cells immortalization along with the human B cell growth factors, Interleukin 2 and Interleukin 4 (1000U/ml of each). Human B cell lines were made in 96 wells round bottom wells. After two weeks, supernatants from these clones were screened by Enzyme Linked Immunosorbent Assay (ELISA), Plaque reduction neutralization test (PRNT) and Cytopathic Effect assay (CPE) to analyze binding/neutralizing activity for dengue virus. B cell lines producing positive antibodies were used as a source of mRNA for

antibody heavy and light chains gene amplification. The heavy and light chains sequences of the antibody were cloned into an in-house pCMV vector and transfected into Freestyle® 293F cells to produce high concentrations of recombinant antibody. Using this methodology, we have cloned and expressed a recombinant antibody that is exquisitely dengue serotype 1 specific and has broad specificity for various Dengue serotype 1 genotypes. This antibody does not bind to other viruses in the Flavivirus genus and, as such, exhibits little or no enhancement of infection of macrophages to other flaviviruses beyond those expected for dengue serotype 1. *In vivo* experiments have shown remarkable prophylactic and therapeutic efficacy in a mouse model of dengue infection. As such, this antibody represents the best available candidate therapeutic for dengue 1 infection in existence.

DEFINITIONS

[0056] It is to be understood that this invention is not limited to particular methods, reagents, compounds, compositions or biological systems, which can, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular aspects only, and is not intended to be limiting. As used in this specification and the appended claims, the singular forms "a", "an" and "the" include plural references unless the content clearly dictates otherwise.

[0057] The term "about" as used herein when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of ±20% or ±10%, more preferably ±5%, even more preferably ±1%, and still more preferably ±0.1% from the specified value, as such variations are appropriate to perform the disclosed methods.

[0058] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although any methods and materials similar or equivalent to those described herein can be used in the practice for testing of the present invention, the preferred materials and methods are described herein.

[0059] "Vertebrate," "mammal," "subject," "mammalian subject," or "patient" are used interchangeably and refer to mammals such as human patients and non-human primates, as well as experimental animals such as rabbits, rats, and mice, cows, horses, goats, and other animals. Animals include all vertebrates, *e.g.*, mammals and non-mammals, such as mice, sheep, dogs, cows, avian species, ducks, geese, pigs, chickens, amphibians, and reptiles.

[0060] "Treating" or "treatment" refers generally to either (i) the prevention of infection or reinfection, *e.g.*, prophylaxis, or (ii) the reduction or elimination of symptoms of a disease

of interest, *e.g.*, therapy. Treating a subject with the compositions of the invention can prevent or reduce the risk of infection from Dengue virus, particularly serotype 1. Treatment can be prophylactic (to prevent or delay the onset of the disease, or to prevent the manifestation of clinical or subclinical symptoms thereof) or therapeutic suppression or alleviation of symptoms after the manifestation of the disease.

[0061] "Preventing" or "prevention" refers to prophylactic administration with compositions of the invention.

[0062] "Therapeutically-effective amount" or "an amount effective to reduce or eliminate infection" or "an effective amount" refers to an amount of an antibody composition that is sufficient to prevent Dengue viral infection or to alleviate (*e.g.*, mitigate, decrease, reduce) at least one of the symptoms associated with such an infection. It is not necessary that the administration of the composition eliminate the symptoms of Dengue infection, as long as the benefits of administration of the composition outweigh the detriments. Likewise, the terms "treat" and "treating" in reference to Dengue infection, as used herein, are not intended to mean that the subject is necessarily cured of infection or that all clinical signs thereof are eliminated, only that some alleviation or improvement in the condition of the subject is effected by administration of the composition.

[0063] "Passive immunity" refers generally to the transfer of active humoral immunity in the form of pre-made antibodies from one individual to another. Thus, passive immunity is a form of short-term immunization that can be achieved by the transfer of antibodies, which can be administered in several possible forms, for example, as human or animal blood plasma or serum, as pooled animal or human immunoglobulin for intravenous (IVIG) or intramuscular (IG) use, as high-titer animal or human IVIG or IG from immunized subjects or from donors recovering from a disease, and as monoclonal antibodies. Passive transfer can be used prophylactically for the prevention of disease onset, as well as, in the treatment of several types of acute infection. Typically, immunity derived from passive immunization lasts for only a short period of time, and provides immediate protection, but the body does not develop memory, therefore the patient is at risk of being infected by the same pathogen later.

ANTIBODIES

[0064] As used herein, the term "antibody" refers to any immunoglobulin or intact molecule as well as to fragments thereof that bind to a specific epitope. Such antibodies include, but are not limited to polyclonal, monoclonal, chimeric, humanized, single chain,

Fab, Fab', F(ab)' fragments and/or F(v) portions of the whole antibody and variants thereof. All isotypes are encompassed by this term, including IgA, IgD, IgE, IgG, and IgM.

[0065] As used herein, the term "antibody fragment" refers specifically to an incomplete or isolated portion of the full sequence of the antibody which retains the antigen binding function of the parent antibody. Examples of antibody fragments include Fab, Fab', F(ab')2, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

[0066] An intact "antibody" comprises at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as HCVR or V_H) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, CH_1, CH_2 and CH_3. Each light chain is comprised of a light chain variable region (abbreviated herein as LCVR or V_L) and a light chain constant region. The light chain constant region is comprised of one domain, C_L. The V_H and V_L regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each V_H and V_L is composed of three CDRs and four FRs, arranged from amino-terminus to carboxyl-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the antibodies can mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (*e.g.*, effector cells) and the first component (Clq) of the classical complement system. The term antibody includes antigen-binding portions of an intact antibody that retain capacity to bind. Examples of binding include (i) a Fab fragment, a monovalent fragment consisting of the V_L, V_H, C_L and CH1 domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CH1 domains; (iv) a Fv fragment consisting of the V_L and V_H domains of a single arm of an antibody, (v) a dAb fragment (Ward *et al.*, Nature, 341:544-546 (1989)), which consists of a VH domain; and (vi) an isolated complementarity determining region (CDR).

[0067] As used herein, the term "single chain antibodies" or "single chain Fv (scFv)" refers to an antibody fusion molecule of the two domains of the Fv fragment, V_L and V_H. Although the two domains of the Fv fragment, V_L and V_H, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the V_L and V_H regions pair to form monovalent

molecules (known as single chain Fv (scFv); see, *e.g.*, Bird *et al.*, Science, 242:423-426 (1988); and Huston *et al.*, Proc Natl Acad Sci USA, 85:5879-5883 (1988)). Such single chain antibodies are included by reference to the term "antibody" fragments and can be prepared by recombinant techniques or enzymatic or chemical cleavage of intact antibodies.

[0068] As used herein, the term "human sequence antibody" includes antibodies having variable and constant regions (if present) derived from human germline immunoglobulin sequences. The human sequence antibodies of the invention can include amino acid residues not encoded by human germline immunoglobulin sequences (*e.g.*, mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*). Such antibodies can be generated in non-human transgenic animals, *e.g.*, as described in PCT App. Pub. Nos. WO 01/14424 and WO 00/37504. However, the term "human sequence antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences (*e.g.*, humanized antibodies).

[0069] Also, recombinant immunoglobulins can be produced. See, Cabilly, U.S. Patent No. 4,816,567, incorporated herein by reference in its entirety and for all purposes; and Queen *et al.*, Proc Natl Acad Sci USA, 86:10029-10033 (1989).

[0070] As used herein, the term "monoclonal antibody" refers to a preparation of antibody molecules of single molecular composition. A monoclonal antibody composition displays a single binding specificity and affinity for a particular epitope. Accordingly, the term "human monoclonal antibody" refers to antibodies displaying a single binding specificity which have variable and constant regions (if present) derived from human germline immunoglobulin sequences. In one aspect, the human monoclonal antibodies are produced by a hybridoma which includes a B cell obtained from a transgenic non-human animal, *e.g.*, a transgenic mouse, having a genome comprising a human heavy chain transgene and a light chain transgene fused to an immortalized cell.

[0071] As used herein, the term "antigen" refers to a substance that prompts the generation of antibodies and can cause an immune response. It can be used interchangeably in the present disclosure with the term "immunogen". In the strict sense, immunogens are those substances that elicit a response from the immune system, whereas antigens are defined as substances that bind to specific antibodies. An antigen or fragment thereof can be a molecule (*i.e.*, an epitope) that makes contact with a particular antibody. When a protein or a fragment of a protein is used to immunize a host animal, numerous regions of the protein can

induce the production of antibodies (*i.e.*, elicit the immune response), which bind specifically to the antigen (given regions or three-dimensional structures on the protein).

[0072] As used herein, the term "humanized antibody," refers to at least one antibody molecule in which the amino acid sequence in the non-antigen binding regions and/or the antigen-binding regions has been altered so that the antibody more closely resembles a human antibody, and still retains its original binding ability.

[0073] In addition, techniques developed for the production of "chimeric antibodies" (Morrison, *et al.*, Proc Natl Acad Sci, 81:6851-6855 (1984), incorporated herein by reference in their entirety) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. For example, the genes from a mouse antibody molecule specific for an autoinducer can be spliced together with genes from a human antibody molecule of appropriate biological activity. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region.

[0074] In addition, techniques have been developed for the production of humanized antibodies (see, *e.g.*, U.S. Patent No. 5,585,089 and U.S. Patent No. 5,225,539, which are incorporated herein by reference in their entirety). An immunoglobulin light or heavy chain variable region consists of a "framework" region interrupted by three hypervariable regions, referred to as complementarity determining regions (CDRs). Briefly, humanized antibodies are antibody molecules from non-human species having one or more CDRs from the non-human species and a framework region from a human immunoglobulin molecule.

[0075] Alternatively, techniques described for the production of single chain antibodies can be adapted to produce single chain antibodies against an immunogenic conjugate of the present disclosure. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide. Fab and F(ab')2 portions of antibody molecules can be prepared by the proteolytic reaction of papain and pepsin, respectively, on substantially intact antibody molecules by methods that are well-known. See *e.g.*, U.S. Patent No. 4,342,566. Fab' antibody molecule portions are also well-known and are produced from F(ab')2 portions followed by reduction of the disulfide bonds linking the two heavy chain portions as with mercaptoethanol, and followed by alkylation of the resulting protein mercaptan with a reagent such as iodoacetamide.

ANTIBODY ASSAYS

[0076] A number of screening assays are known in the art for assaying antibodies of interest to confirm their specificity and affinity and to determine whether those antibodies cross-react with other proteins.

[0077] The terms "specific binding" or "specifically binding" refer to the interaction between the antigen and their corresponding antibodies. The interaction is dependent upon the presence of a particular structure of the protein recognized by the binding molecule (*i.e.*, the antigen or epitope). In order for binding to be specific, it should involve antibody binding of the epitope(s) of interest and not background antigens.

[0078] Once antibodies are produced, they are assayed to confirm that they are specific for the antigen of interest and to determine whether they exhibit any cross reactivity with other antigens. One method of conducting such assays is a sera screen assay as described in U.S. App. Pub. No. 2004/0126829, the contents of which are hereby expressly incorporated herein by reference. However, other methods of assaying for quality control are within the skill of a person of ordinary skill in the art and therefore are also within the scope of the present disclosure.

[0079] Antibodies, or antigen-binding fragments, variants or derivatives thereof of the present disclosure can also be described or specified in terms of their binding affinity to an antigen. The affinity of an antibody for an antigen can be determined experimentally using any suitable method. (See, *e.g.*, Berzofsky *et al.*, "Antibody-Antigen Interactions," In Fundamental Immunology, Paul, W. E., Ed., Raven Press: New York, N.Y. (1984); Kuby, Janis Immunology, W. H. Freeman and Company: New York, N.Y. (1992); and methods described herein). The measured affinity of a particular antibody-antigen interaction can vary if measured under different conditions (*e.g.*, salt concentration, pH). Thus, measurements of affinity and other antigen-binding parameters (*e.g.*, K_D, K_a, K_d) are preferably made with standardized solutions of antibody and antigen, and a standardized buffer.

[0080] The affinity binding constant (K_{aff}) can be determined using the following formula:

$$K_{aff} = \frac{(n-1)}{2\left(n\left[mAb'\right]_t - \left[mAb\right]_t\right)}$$

in which:

$$n = \frac{\left[mAg\right]_t}{\left[mAg'\right]_t}$$

[0081] [*mAb*] is the concentration of free antigen sites, and [*mAg*] is the concentration of free monoclonal binding sites as determined at two different antigen concentrations (*i.e.*, $[mAg]_t$ and $[mAg']_t$) (Beatty *et al.*, J Imm Meth, 100:173-179 (1987)).

[0082] The term "high affinity" for an antibody refers to an equilibrium association constant (K_{aff}) of at least about 1×10^7 liters/mole, or at least about 1×10^8 liters/mole, or at least about 1×10^9 liters/mole, or at least about 1×10^{10} liters/mole, or at least about 1×10^{11} liters/mole, or at least about 1×10^{12} liters/mole, or at least about 1×10^{13} liters/mole, or at least about 1×10^{14} liters/mole or greater. "High affinity" binding can vary for antibody isotypes. K_D, the equilibrium dissociation constant, is a term that is also used to describe antibody affinity and is the inverse of K_{aff}.

[0083] K_D, the equilibrium dissociation constant, is a term that is also used to describe antibody affinity and is the inverse of K_{aff}. If K_D is used, the term "high affinity" for an antibody refers to an equilibrium dissociation constant (K_D) of less than about 1×10^{-7} mole/liters, or less than about 1×10^{-8} mole/liters, or less than about 1×10^{-9} mole/liters, or less than about 1×10^{-10} mole/liters, or less than about 1×10^{-11} mole/liters, or less than about 1×10^{-12} mole/liters, or less than about 1×10^{-13} mole/liters, or less than about 1×10^{-14} mole/liters or lower.

[0084] The production of antibodies according to the present disclosure provides for antibodies with the characteristics of those produced in the course of a physiological human immune response, i.e. antibody specificities that can only be selected by the human immune system. In the present case, this includes a response to the human pathogen Dengue virus, serotype 1. In some embodiments, antibodies of the present disclosure possess the characteristics of those produced in the course of a response to infection by Dengue virus. These antibodies can be used as prophylactic or therapeutic agents upon appropriate formulation.

[0085] In relation to a particular pathogen, a "neutralizing antibody", "broadly neutralizing antibody", or "neutralizing monoclonal antibody", all of which are used interchangeably herein, is one that can neutralize the ability of that pathogen to initiate and/or perpetuate an infection in a host. In some embodiments, monoclonal antibodies produced in accordance with the present disclosure have neutralizing activity, where the antibody can neutralize at a concentration of 10^{-9}M or lower (e.g. 10^{-10}M, 10^{-11}M, 10^{-12}M or lower).

[0086] The immunoglobulin molecules of the present invention can be of any type (e.g., IgG, IgE, IgM, IgD, IgA and IgY), class (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2), or subclass of immunoglobulin molecule. In some embodiments, the antibodies are antigen-

binding antibody fragments (e.g., human) and include, but are not limited to, Fab, Fab' and $F(ab')_2$, Fd, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (sdFv) and fragments comprising either a V_L or V_H domain. Antigen-binding antibody fragments, including single-chain antibodies, can comprise the variable region(s) alone or in combination with the entirety or a portion of the following: hinge region, CH1, CH2, and CH3 domains. Also included in the present disclosure are antigen-binding fragments comprising any combination of variable region(s) with a hinge region, CH1, CH2, and CH3 domains.

B CELL ISOLATION

[0087] As used herein, the terms "B cell", "B memory cell", "B lymphocyte", "B memory lymphocyte", "memory cells", "memory B cell", and variants thereof are used interchangeably and refer to B cells of the humoral immune response. As understood in the art, B cells are lymphocytes that play a role in the humoral immune response (as opposed to the cell-mediated immune response, which is governed by T cells). At least one function of B cells is to make antibodies against antigens, perform the role of Antigen Presenting Cells (APCs) and eventually develop into memory B cells after activation by antigen interaction. B cells are a component of the adaptive immune system.

[0088] The phrase "primary B cell" can refer in some embodiments to a B cell taken directly from a living organism (*e.g.*, a human). In some embodiments, a primary B cell can be cultured in a primary cell culture. A primary B cell can be derived, obtained or collected from a subject in any manner known to those of skill in the art. In some embodiments, a primary B cell is obtained from a subject infected with or possessing an antigen of interest.

[0089] The methods of the present disclosure can be applied for the identification of monoclonal antibodies expressed by human B cells selected from donors, such as patients exposed to an infective agent, e.g., Dengue virus. Thus, the donor can be naive, vaccinated, affected by one or more diseases or infections, already exposed and/or resistant to specific therapeutic treatments, presenting a specific clinical index or status, inadvertently exposed to a pathogen, etc.

[0090] A donor's sera can be used as such for an initial determination of their seropositivity to an antigen, since the specificity and long-term maintenance of the adaptive immune responses (even years after the last exposure to this antigen) may allow a qualitative determination that is sufficient for selecting donors. The nature and sensitivity of the screening assay used is critical in identifying the most suitable donor and, preferably, the

assay used to screen donor serum should be the same as that used to screen supernatants from immortalized antibody-secreting B cells and designed to detect an antibody with the desired functional activity (i.e., neutralization activity).

[0091] The choice of the tissue or the organ from which the cells are purified may be dictated by the availability of appropriate cells in sufficient amount. Cells can be obtained from fresh or frozen samples and/or from samples obtained from a number of individuals that have been pooled to provide enough starting material.

[0092] A preliminary screen can be done on a panel of candidate donors, using samples containing antibody-secreting cells (such as total peripheral blood or serum). In particular, mononuclear cells can be isolated from blood or lymphatic tissues using standard separation techniques for isolating peripheral blood mononuclear cells (PBMCs), such as gradient centrifugation. After and/or before this separation step, the samples of sera (or plasma), cell culture supernatants, or cells (obtained from different patients, from different tissues, and/or at different time points) can be pre-screened using standard technologies for detecting the presence of antibodies and antibody-secreting cells (e.g. ELISA, BIACORE, Western blot, FACS, SERPA, antigen arrays, neutralization of viral infection in a cell culture system, or ELISPOT assays).

[0093] Examples in the art include, for example, the use of ELISPOT for characterizing the immune response in vaccinated donors (Crotty S et al., 2004), the use of antigen microarrays as diagnostic tools for newly infected patients (Mezzasoma L et al., 2002), and other technologies for measuring antigen-specific immune responses (Kern F et al., 2005).

[0094] This preliminary qualitative analysis of antibody response to the therapeutic target should allow the identification of donors having B cells expressing higher antibody titers directed to the desired purified antigen (e.g. a specific recombinant viral protein), a mixture of related antigens (e.g. obtained from partially purified viral preparation), or a bioassay (e.g. neutralization of viral infectivity).

[0095] Once one or more donors are selected, the source of B cells can be spleen, blood, lymph nodes, bone marrow, tumor infiltrating lymphocytes, lymphocytes from sites of chronic infection/inflammation. However, peripheral blood is usually easier to obtain from donors, to store, and to monitor for the serological response against an antigen over a defined period of time.

[0096] For example, starting from 5-50 ml of peripheral blood, approximately 10-100 million of PBMCs (peripheral blood mononuclear cells) can be purified, a number of cells

that would allow a sufficiently large population of antibody-secreting cells to be screened after being immortalized using the methods disclosed herein.

[0097] After the isolation of PBMCs from biological samples, a specific selection of antibody-secreting cells can be performed, using methods known in the art, on the basis of the expression of cell surface markers on their surface and, if appropriate, of other proteins, as well as the proliferation activity, the metabolic and/or morphological status of the cells.

[0098] In particular, various technologies for the purification of antibody-secreting cells from human samples make use of different means and conditions for positive or negative selection. These cells can be efficiently selected by physically separating those expressing cell surface markers specific for cells that express and secrete antibodies (e.g. human B cells). Specific protocols can be found in the art (see, *e.g.*, Callard R and Kotowicz K "Human B-cell responses to cytokines" in Cytokine Cell Biology: A practical Approach. Balkwill F. (ed.) Oxford University Press, 2000, pg. 17-31).

[0099] The selection can be performed using antibodies that bind specifically to one of these cell surface proteins and that can be linked to solid supports (e.g. microbeads or plastic plates) or labeled with a fluorochrome that can be detected using fluorescence-activated cell sorters (FACS). For example, human B cells have been selected on the basis of their affinity for supports (such as microbeads) binding CD19, CD27, and/or CD22 microbeads, or for the lack of binding affinity for antibodies specific for certain isotypes prior to EBV immortalization (Li H et al., 1995, Bemasconi N et al., 2003; Traggiai E et al., 2004).

[00100] As shown herein, CD22, which is a B-cell restricted transmembrane protein that controls signal transduction pathways related to antigen recognition and B cell activation (Nitschke L, 2005), can be used for the initial B cell selection. Since the CD22 positive population contains cells that express antibodies having different isotypes and specificities, other cell surface markers can also be used for selecting the cells.

[00101] Alternatively or additionally, a specific enrichment of antibody-secreting cells can be obtained by applying a CD27-based selection in addition to the CD22-based selection. CD27 is known to be a marker for human B cells that have somatically mutated variable region genes (Borst J et al., 2005). Additional markers such as CD5, CD24, CD25, CD86, CD38, CD45, CD70, or CD69 could be used to either deplete or enrich for the desired population of cells. Thus, depending on the donor's history of exposure to the antigen (e.g. viral, bacterial, parasite), the antibody titer, total B cells, CD22 enriched B cells, or further enriched B cell subpopulations such as CD27 positive B cells can be used.

EBV TRANSFORMATION OF B CELLS

[00102] The selected and stimulated population of cells that express antibodies having specific isotypes can be immortalized using a viral immortalizing agent. Different immortalizing agents can be used on antibody-secreting cells to obtain immortalized antibody-secreting cells.

[00103] Among the viral immortalizing agents, a virus that infects and immortalizes antibody-secreting cells can be preferably used in the practice of the invention. Commonly used viruses are lymphotropic viruses, grouped in the gamma class of herpesvirus. Members of this virus family infect lymphocytes in a species-specific manner, and are associated with lymphoproliferative disorders and the development of several malignancies (Nicholas J, 2000; Rickinson A, 2001).

[00104] EBV (Epstein-Barr virus, also known as herpesvirus 4), and HHV-8 (human herpesvirus 8, also known as KSHV, Kaposi's Sarcoma associated Herpervirus) infect and immortalize human lymphocytes. MHV-68 (murine herpesvirus 68), HVS (herpesvirus Samiri), RRV (Rhesus Rhadinovirus), LCV (primate Lymphocryptovirus), EHV-2 (Equine Herpesvirus 2) HVA (Herpesvirus Ateles), and AHV-1 (Alcelaphine Herpesvirus 1) are other oncogenic, lymphotropic herpesvirus having common genetic features conserved among them and similar pathogenic effects in different mammalian host cells. These viruses can be used in practice of the present invention.

[00105] In addition to the use of intact viruses, recombinant DNA constructs that contain specific viral proteins have been successfully used to immortalize B cells (Damania B 2004; Kilger E et al., 1998). Vectors containing viral genes can be transduced into cells, sometimes making use of retroviral systems or packaging cell lines which provide all the necessary factors in trans for the formation of such virus-like particles, can also be used in the methods of the invention.

[00106] EBV-mediated immortalization is a complex process involving the immortalization of B cells due to proteins that are expressed by EBV, and is regulated by the interaction between EBV and host cells proteins (Sugimoto M et al., 2004; Bishop G E, and Busch L K, 2002). If desired, the immortalization process can be followed by measuring the expression of specific EBV proteins and transcripts such as EBNA2, EBNA1, LMP2, LMP1, or EBERs (Thorley-Lawson D A, 2001). These proteins can be detected by PCR, immunofluorescence, Western blot, or other methods allowing the detection of EBV DNA

and proteins in infected cells (Schlee M et al., 2004; Park C H et al., 2004; Humme S et al., 2003; Konishi K et al., 2001; Haan K et al., 2001).

SCREENING AND ISOLATION OF TRANSFORMED B CELLS

[00107] In some embodiments, transformed and/or activated B cells can be screened for those having the desired antigen specificity, and individual B cell clones can then be produced from the positive cells. The screening step can be carried out by ELISA, by staining of tissues or cells (including transfected cells), a neutralization assay, and/or one of a number of other methods known in the art for identifying desired antigen specificity. The assay can select on the basis of simple antigen recognition, or can select on the additional basis of a desired function, e.g. neutralizing antibodies rather than just antigen-binding antibodies.

[00108] In some embodiments, a cloning step for separating individual clones from the mixture of positive cells can be carried out using limiting dilution, micromanipulation, single cell deposition by cell sorting, and/or by any other method known in the art. In some embodiments, cloning is carried out using limiting dilution. In some embodiments, cloned B cells are derived from B cells that have been immortalized using EBV-transformation coupled with inhibition of host innate response to activator-mediated proliferative signals.

[00109] In some embodiments, the present disclosure provides for the production of immortalized B cells that produce antibodies having a desired antigen specificity. Such B cells can be used in various ways, e.g. as a source of monoclonal antibodies, as a source of nucleic acid (DNA or mRNA) encoding a monoclonal antibody of interest, for delivery to subjects for cellular therapy, as a therapeutic or pharmaceutical.

[00110] In some embodiments, the supernatant from the activated B cells in culture can be screened for antibodies of interest using known methods known in the art. Screening is performed to identify one or more monoclonal antibodies capable of binding to an antigen of interest. Such screening can be performed on culture supernatant and/or purified antibodies. Alternatively, screening can be carried out using culture supernatant and/or purified antibodies from activated and/or immortalized B cells. In addition, where cross-reactive antibodies are of interest, the ability of the monoclonal antibodies to cross-react with two or more different antigens can be determined. Moreover, in some embodiments, it can be desirable to screen for antibodies with certain functional characteristics (e.g. neutralizing activity).

[00111] The binding specificity of monoclonal antibodies produced by the present disclosure can, for example, be determined in an immunoassay, e.g. by immunoprecipitation

or other in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoadsorbent assay (ELISA).

[00112] Representative general classes of screening methods that can be employed include, but are not limited to, (a) antibody capture assays; (b) antigen capture assays; and (c) functional screens.

[00113] In antibody capture assays, the antigen can be bound to a solid phase, monoclonal antibodies to be tested are allowed to bind to the antigen, unbound antibodies are removed by washing, and then the bound antibodies are detected, e.g. by a secondary reagent such as a labeled antibody that specifically recognizes the antibody.

[00114] For an antigen capture assay, the antigen can be labeled directly. In one embodiment, monoclonal antibodies to be tested can be bound to a solid phase and then reacted with the optionally labeled antigen. Alternatively, the antibody-antigen complex can be allowed to form by immunoprecipitation prior to binding of the monoclonal antibody to be tested to a solid phase. Once the antibody-antigen complexes are bound to the solid phase, unbound antigen can be removed by washing and positives can be identified by detecting the antigen.

[00115] Various functional screens exist for identifying monoclonal antibodies with desired activities. In the present disclosure, one such screen, as described in the Examples, is a neutralization assay.

RECOMBINANT EXPRESSION

[00116] The methods of the present disclosure also provide for obtaining and/or sequencing a nucleic acid for the antibody from the selected B cell clone; and utilizing the nucleic acid to generate a host cell that can express the antibody of interest.

[00117] In some embodiments, the nucleotide sequence encoding a desired antibody can be sequenced and thereafter employed in a heterologous expression system, e.g. 293 cells or CHO cells. In some embodiments, an antibody can be recombinantly expressed by obtaining one or more nucleic acids (e.g. heavy and/or light chain genes) from the a B cell clone that encodes the antibody of interest and inserting the nucleic acid into a host cell in order to permit expression of the antibody of interest in that host.

[00118] Production of antibodies using recombinant DNA methods is described, for example, in U.S. Pat. No. 4,816,567. For recombinant production of the antibody, the nucleic acid encoding it is isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. DNA encoding a monoclonal antibody is

readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the antibody). Vectors that can be used generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence. Examples of such expression system components are disclosed in, for example, U.S. Pat. No. 5,739,277. Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells (see, e.g., U.S. Pat. No. 5,739,277).

PHARMACEUTICAL COMPOSITIONS

[00119] The presently disclosed subject matter provides pharmaceutical compositions comprising the antibodies produced in accordance with the present disclosure. In some embodiments, pharmaceutical compositions comprising transformed and/or activated B cells are provided. In some embodiments, a pharmaceutical composition can comprise one or more monoclonal antibodies produced in using the methods disclosed herein. In some embodiments, both monoclonal antibodies as well as the transformed and/or activated B cells of the presently disclosed subject matter can be included in a pharmaceutical composition. In some embodiments, a panel of monoclonal antibodies produced according to the present disclosure can be included in a pharmaceutical composition. In some embodiments, the monoclonal antibodies and/or B cells produced according to the present disclosure can be included with one or more additional agents, for example, antiviral drugs or analgesics.

[00120] In some embodiments a pharmaceutical composition can also contain a pharmaceutically acceptable carrier or adjuvant for administration of the antibody. In some embodiments, the carrier is pharmaceutically acceptable for use in humans. The carrier or adjuvant should not itself induce the production of antibodies harmful to the individual receiving the composition and should not be toxic. Suitable carriers can be large, slowly metabolized macromolecules such as proteins, polypeptides, liposomes, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, ammo acid copolymers and inactive virus particles.

[00121] Pharmaceutically acceptable salts can be used, for example mineral acid salts, such as hydrochlorides, hydrobromides, phosphates and sulphates, or salts of organic acids, such as acetates, propionates, malonate and benzoates.

[00122] Pharmaceutically acceptable carriers in therapeutic compositions can additionally contain liquids such as water, saline, glycerol and ethanol. Additionally, auxiliary substances,

such as wetting or emulsifying agents or pH buffering substances, can be present in such compositions. Such carriers enable the pharmaceutical compositions to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries and suspensions, for ingestion by the patient.

[00123] The compositions of the presently disclosed subject matter can further comprise a carrier to facilitate composition preparation and administration. Any suitable delivery vehicle or carrier can be used, including but not limited to a microcapsule, for example a microsphere or a nanosphere (Manome et al. (1994) Cancer Res 54:5408-5413; Saltzman & Fung (1997) Adv Drug Deliv Rev 26:209-230), a glycosaminoglycan (U.S. Pat. No. 6,106,866), a fatty acid (U.S. Pat. No. 5,994,392), a fatty emulsion (U.S. Pat. No. 5,651,991), a lipid or lipid derivative (U.S. Pat. No. 5,786,387), collagen (U.S. Pat. No. 5,922,356), a polysaccharide or derivative thereof (U.S. Pat. No. 5,688,931), a nanosuspension (U.S. Pat. No. 5,858,410), a polymeric micelle or conjugate (Goldman et al. (1997) Cancer Res 57:1447-1451 and U.S. Pat. Nos. 4,551,482, 5,714,166, 5,510,103, 5,490,840, and 5,855,900), and a polysome (U.S. Pat. No. 5,922,545).

[00124] Antibody sequences can be coupled to active agents or carriers using methods known in the art, including but not limited to carbodiimide conjugation, esterification, sodium periodate oxidation followed by reductive alkylation, and glutaraldehyde crosslinking (Goldman et al. (1997) Cancer Res. 57:1447-1451; Cheng (1996) Hum. Gene Ther. 7:275-282; Neri et al. (1997) Nat. Biotechnol. 15:1271-1275; Nabel (1997) Vectors for Gene Therapy. In Current Protocols in Human Genetics, John Wiley & Sons, New York; Park et al. (1997) Adv. Pharmacol. 40:399-435; Pasqualini et al. (1997) Nat. Biotechnol. 15:542-546; Bauminger & Wilchek (1980) Meth. Enzymol. 70:151-159; U.S. Pat. No. 6,071,890; and European Patent No. 0 439 095).

[00125] A therapeutic composition of the present invention comprises in some embodiments a pharmaceutical composition that includes a pharmaceutically acceptable carrier. Suitable formulations include aqueous and non-aqueous sterile injection solutions which can contain anti-oxidants, buffers, bacteriostats, bactericidal antibiotics and solutes which render the formulation isotonic with the bodily fluids of the intended recipient; and aqueous and non-aqueous sterile suspensions which can include suspending agents and thickening agents. The formulations can be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and can be stored in a frozen or freeze-dried (lyophilized) condition requiring only the addition of sterile liquid carrier, for example water for injections, immediately prior to use. Some exemplary ingredients are SDS in the range of

in some embodiments 0.1 to 10 mg/ml, in some embodiments about 2.0 mg/ml; and/or mannitol or another sugar in the range of in some embodiments 10 to 100 mg/ml, in some embodiments about 30 mg/ml; and/or phosphate-buffered saline (PBS). Any other agents conventional in the art having regard to the type of formulation in question can be used. In some embodiments, the carrier is pharmaceutically acceptable. In some embodiments the carrier is pharmaceutically acceptable for use in humans.

[00126] Pharmaceutical compositions of the present disclosure can have a pH between 5.5 and 8.5, preferably between 6 and 8, and more preferably about 7. The pH can be maintained by the use of a buffer. The composition can be sterile and/or pyrogen free. The composition can be isotonic with respect to humans. Pharmaceutical compositions of the presently disclosed subject matter can be supplied in hermetically-sealed containers.

[00127] Pharmaceutical compositions can include an effective amount of one or more antibodies as described herein. In some embodiments, a pharmaceutical composition can comprise an amount that is sufficient to treat, ameliorate, or prevent a desired disease or condition, or to exhibit a detectable therapeutic effect. Therapeutic effects also include reduction in physical symptoms. The precise effective amount for any particular subject will depend upon their size and health, the nature and extent of the condition, and therapeutics or combination of therapeutics selected for administration. The effective amount for a given situation is determined by routine experimentation as practiced by one of ordinary skill in the art.

TREATMENT REGIMENS: PHARMACOKINETICS

[00128] The pharmaceutical compositions of the invention can be administered in a variety of unit dosage forms depending upon the method of administration. Dosages for typical antibody pharmaceutical compositions are well known to those of skill in the art. Such dosages are typically advisory in nature and are adjusted depending on the particular therapeutic context or patient tolerance. The amount antibody adequate to accomplish this is defined as a "therapeutically effective dose." The dosage schedule and amounts effective for this use, *i.e.*, the "dosing regimen," will depend upon a variety of factors, including the stage of the disease or condition, the severity of the disease or condition, the general state of the patient's health, the patient's physical status, age, pharmaceutical formulation and concentration of active agent, and the like. In calculating the dosage regimen for a patient, the mode of administration also is taken into consideration. The dosage regimen must also take into consideration the pharmacokinetics, *i.e.*, the pharmaceutical composition's rate of

absorption, bioavailability, metabolism, clearance, and the like. See, *e.g.*, the latest Remington's; Egleton, *Peptides* 18: 1431-1439, 1997; Langer, *Science* 249: 1527-1533, 1990.

[00129] For purposes of the present invention, a therapeutically effective amount of a composition comprising an antibody, contains about 0.05 to 1500 μg protein, preferably about 10 to 1000 μg protein, more preferably about 30 to 500 μg and most preferably about 40 to 300 pg, or any integer between these values. For example, antibodies of the invention can be administered to a subject at a dose of about 0.1 μg to about 200 mg, e.g., from about 0.1 μg to about 5 μg, from about 5 μg to about 10 μg, from about 10 μg to about 25 μg, from about 25 μg to about 50 μg, from about 50 μg to about 100 μg, from about 100 μg to about 500 μg, from about 500 μg to about 1 mg, from about 1 mg to about 2 mg, with optional boosters given at, for example, 1 week, 2 weeks, 3 weeks, 4 weeks, two months, three months, 6 months and/or a year later. It is understood that the specific dose level for any particular patient depends upon a variety of factors including the activity of the specific antibody employed, the age, body weight, general health, sex, diet, time of administration, route of administration, and rate of excretion, drug combination and the severity of the particular disease undergoing therapy.

[00130] Routes of administration include, but are not limited to, oral, topical, subcutaneous, intramuscular, intravenous, subcutaneous, intradermal, transdermal and subdermal. Depending on the route of administration, the volume per dose is preferably about 0.001 to 10 ml, more preferably about 0.01 to 5 ml, and most preferably about 0.1 to 3 ml. Compositions can be administered in a single dose treatment or in multiple dose treatments on a schedule and over a time period appropriate to the age, weight and condition of the subject, the particular antibody formulation used, and the route of administration.

KITS

[00131] The invention provides kits comprising antibodies produced in accordance with the present disclosure which can be used, for instance, for therapeutic applications described above. The article of manufacture comprises a container with a label. Suitable containers include, for example, bottles, vials, and test tubes. The containers can be formed from a variety of materials such as glass or plastic. The container holds a composition which includes an active agent that is effective for therapeutic applications, such as described above. The active agent in the composition can comprise the antibody. The label on the container indicates that the composition is used for a particular therapy or non-therapeutic application,

and can also indicate directions for either *in vivo* or *in vitro* use, such as those described above.

[00132] The following examples of specific aspects for carrying out the present invention are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

EXAMPLES

Methods and materials

Ethics Statement

[00133] Informed consent was obtained and all procedures carried out under an approved protocol from the National University Institutional Review Board (NUS-IRB number is 06-196).

Cells and viruses

[00134] C6/36 cells and BHK-21 cells were cultured as described previously (28). All dengue strains except EHI and PVP 159 strains were obtained from Novartis Institute of Tropical Diseases, Singapore (NITD). EHI strain was obtained from Environmental Health Institute, Singapore (EHI) and PVP 159 (DENV1/SG/07K3640DK1/2008) from the EDEN patient cohort (29).

Cloning of B cells

[00135] Isolation and immortalization of B cells was carried out as described previously (*10*). After 15 days of culture, supernatants were screened for DENV-specific antibodies by ELISA and PRNT.

ELISA binding assays

[00136] 96 well flat bottom plates (Maxisorp plates, Nunc) were coated with mouse 4G2 antibody overnight at 5 μg/ml overnight. Plates were washed three times with PBS/Tween-20 0.01%. Different DENV stains were added at $1x10^5$ pfu in 50 μl per well and further incubated for 2 h. Plates were washed three times with PBS/Tween-20 0.01%. HM14C10 was added to the plates and incubated for a further 1 hr. Plates were washed three times with PBS/Tween-20 0.01%. Anti-human IgG conjugated HRP (Pierce, Singapore) was added and incubated for 1 h. TMB substrate (GE healthcare, Singapore) was added and 0.1 M sulphuric acid used to stop the reaction.

Production of recombinant HM14c10

[00137] RNA from B cells was extracted using an RNA extraction kit (Qiagen). The Cloning and expression of recombinant antibodies was conducted as previously described (30).

Antibody-dependent enhancement assay

[00138] Dengue virus (5 X 10^2 pfu/ml) was pre-incubated with media, individual monoclonal antibodies (HM4G2, HM14c10 or HM14c10 N297Q) or subclasses of HM14c10 monoclonal antibodies (IgG1, IgG2, IgG3 or IgG4) and then added to 10^5 of K562 cells. After an hour, cells were washed extensively with PBS to remove unbound virus and monoclonal antibody. After an additional 48 h, supernatants were harvested and viral titers determined by plaque assay on BHK-21 cells.

In vivo mouse experiments

[00139] AG129 mice are deficient in IFN-α/β and –γ receptors (*31*). The mice were handled in accordance with the Institutional Animal Care and Use Committee recommendations (IACUC protocol no: 018/11). A schematic diagram detailing the prophylactic and therapeutic applications of HM14c10 versus PBS treated controls is provided in Figure 21. Mice were sacrificed and viremia quantified by an established plaque assay (*32*).

Time-lapse Confocal Live Cell Imaging

[00140] All time-lapse live cell microscopy was performed on an inverted A1Rsi confocal microscope (Nikon, Japan) using Plan-Apochromat 100X 1.4 numerical aperture (N.A.) lens. Live cell imaging was performed with living, unfixed BHK cells grown on 25 mm glass coverslips (Marienfeld GmbH, Germany) mounted onto chamber holder (Nikon, Japan). Cells were seeded at a density of 4×10^4/well 1 day before the experiment and cultured in RPMI 1640 supplemented with 10% FCS. For the simultaneous detection of Alexa Fluor-488 labelled antibodies and Alexa Fluor-647 labelled DEN1 viruses, the 488 nm line of an argon ion laser and the light of a 633-nm helium neon laser were directed over an HFT UV/488/633 beam splitter, and fluorescence was detected using an NFT 545 beam splitter in combination with a 505–530 band pass filter for Alexa Fluor-488 detection and an 650 long pass filter for Alexa Fluor-647 detection. Images were captured at 30 sec intervals at 1 frame per sec (fps) for 30 to 60 min. All live cell imaging experiments were performed using cells incubated at 37 °C in 5% CO_2 microscope cage incubator system (OkoLab, Italy). The images were analyzed and processed by Nikon Imaging Software (NIS) elements C software (64 bit, version 3, SP7/build 547) [Nikon, Japan].

Quantification of Intracellular Fluorescence

[00141] The effect of antibody on the endocytosis of DENV1 was evaluated by measuring the relative level of fluorescence within the living cells. After treatment with the respective antibody, images of at least 100 cells were randomly acquired using A1Rsi confocal microscope from three independent experiments. The intracellular region of the cells were then individually demarcated manually using the "region of interest" [ROI] function of NIS Elements software (Nikon, Japan) and the relative fluorescence level of Alexa Fluor-488 within each cell was measured using ROI statistics function of the software. The average, standard deviation and student t-test were calculated for each cell population using Microsoft Excel. The fluorescence from untreated cell populations infected with DEN1 were normalized to 100% and used as a comparison to antibody-treated infected cells.

CryoEM

[00142] Dengue virus (strain PVP 159) was prepared as described previously (*3*). Virus was mixed with Fab HM14c10 in a molar ratio of 1:1, incubated at 37 °C for 30 min, and then 4 °C for 2 h. The complex was then flash frozen in liquid ethane on lacey carbon grids, which were coated with a thin layer of continuous carbon. Virus particles were imaged with a 300 kV FEI Titan Krios in the following conditions: electron dose of 16 $e^{-}/Å^2$, magnification of 47,000, defocus range of 1 μm to 3 μm. The images were recorded on a 4K by 4K Gatan CCD camera resulting in a pixel size of 1.9 Å per pixel. The total of 5,566 particles were boxed and contrast transfer function parameters were determined by using the programs boxer and ctfit, respectively, in the EMAN (*33*) program suite. Orientation of the particles was determined by using multi-path simulated annealing (MPSA) protocol (*34*). West Nile virus was used as an initial model (*26*). The three-dimensional map was generated by using the program make3d in EMAN. The resolution of the final map was found to be 7 Å resolution as determined by the fourier shell coefficient cutoff of 0.5. The DENV1 post-fusion E protein crystal structure (*18*) does not fit well into the cryoEM density map as a rigid body, the domains in the E protein were thus broken up and then fitted separately. The fit of the molecules into the cryoEM map (set at 4σ contour level) were then optimized by using the "fit-in-map" function of Chimera (*35*). To create a homology model of HM14c10 variable region, a structure with the best sequence match was chosen (PDB code 2GHW) and the homology model was created by using the Modeller (*19*). The heavy and light chain of the homology model were fitted separately into the cryoEM map (set at 3σ contour level) in the two possible orientations of the Fab (Fig. 19).

Example 1: Isolation of a strongly neutralizing, DENV1 specific antibody 14c10 from a convalescent DENV1 infected patient.

[00143] A group of B-lymphocyte cell lines secreting antibodies with serotype-specific binding and neutralizing activity for DENV1 were identified, sub-cloned and expanded. One of these cell lines, BCL-14c10, produced IgG with significantly stronger binding and neutralizing activity than others (Fig. 14A). This cell line was used as a source of immunoglobulin gene templates for PCR amplification and expression of recombinant human IgG1s (Fig. 14B(a)). One recombinant human antibody (HM) 14c10 had comparable binding activity for DENV1 with the parental BCL-14c10 (Fig. 14B(b)). HM14c10 neutralized and bound to DENV1 but not DENV2, 3 or 4 (Fig. 14C), and exhibited strong neutralizing activity with a $PRNT_{50}$ of 0.328 μg/ml *in vitro* (Fig. 11A).

[00144] The ADE activity linked to the development of DHF and DSS has been proposed to occur when sub-neutralizing concentrations of antibodies and DENV form complexes that bind to Fc receptor-bearing cells. This leads to an increase in virus uptake and secretion of pro-inflammatory cytokines and chemokines (*11*). We compared the ADE activity of HM14c10 with a humanized anti-Flavivirus monoclonal antibody HM4G2 using an established *in vitro* assay employing the FcγR expressing myelomonocytic cell line K562 (*12*). HM4G2 has cross-serotype binding activity and targets a conserved fusion loop of E-DII on the DENV1-4 (*13*). We observed that HM14c10 exhibits some homotypic enhancement of DENV1 infection at sub-neutralizing concentrations but no enhancing activity for DENV2, 3 or 4, to which it does not bind. In contrast, HM4G2 mediates enhancement of all four serotypes at sub-neutralizing concentrations (Fig. 11B). To investigate the contribution of K562 FcγRs to the observed homotypic ADE activity of HM14c10, we expressed the antibody as a Fab fragment or reduced FcγR binding by removing the glycosylation site on human IgG1 through substitution of the aspargine residue (N) at position 297 for glutamine (Q) (*14*). Both the HM14c10Fab and N297Q mutant exhibited a reduction in their homotypic ADE activity versus whole IgG1 controls (Fig. 11C(a)). We next compared the influence of IgG sub-class on ADE activity and observed a partial correlation with the reported binding activities for FcγRIIA on K562 (*15*). The ADE activity can be ranked as follows: IgG3>IgG1>IgG2>IgG4 with IgG3 being highest and IgG4 the lowest (Fig. 11C(b)). Thus the ADE activity of this neutralizing anti-DENV antibody appears dependent on FcγR binding though it should be noted that the influence of the high affinity FcγR1 and complement components on virus neutralization was not addressed in these experiments (*16*).

[00145] An additional complexity in DENV is the presence of multiple genotypes within a single serotype. DENV1 genotypes can vary up to 3% in their amino acid composition and previous reports of mouse anti-DENV antibodies have suggested that protective activity can vary between genotypes (*17*). We compared the binding activity of HM14c10 for a number of DENV1 clinical isolates representing two disparate DENV1 genotypes (I and IV) with HM4G2. Both HM14c10 and HM4G2 exhibited binding activity for the genotypes tested, with HM4G2 displaying better binding characteristics in all cases (Fig. 15). In contrast, HM14c10 exhibited superior neutralization activity compared to HM4G2 for all of the isolates/genotypes tested (Fig. 11D).

Example 2: HM14c10 binds a quaternary structure dependent epitope.

[00146] The exact nature of the interaction between a given antibody and the DENV must hold the key to explaining neutralization. To determine this, a cryo-electron microscopy (cryoEM) structure of Fab HM14c10:DENV1 complex was solved to 7 Å resolution (Fig. 12A). At full occupancy, 120 copies of Fab HM14c10 bind to all of the available 180 copies of E proteins on the virus surface. To identify the footprint of HM14c10 on E protein, the crystal structure of DENV1 E protein (*18*) was fitted into the cryoEM density map (Fig. 16 and Table 1). The 7 Å resolution cryoEM map showed clear density connections between the HM14c10 Fabs and the E proteins, allowing the identification of E protein residues at the interacting interface (Fig. 12B and Fig. 17). The epitope recognized by HM14c10 is dependent on the quaternary structure of the virus. Two Fabs of HM14c10 bind to three E proteins in the virus asymmetric unit (Fig. 12C and D). Each antibody binds across two adjacent E proteins with half of the epitope on E-DIII and the other half on E-DI and the E-DI-E-DII hinge of a neighboring E protein.

[00147] To understand the Fab interaction with the E protein, a homology model of the variable region of HM14c10 was created (Fig. 18) based on a reference human antibody structure (PDB code 2GHW) by using the Modeller (*19*). The variable region of the light and heavy chain of the homology model were then fitted into the cryoEM densities. Although the structures of both chains are similar, there is a distinctive fit that gives a better correlation to the density (Fig. 19A and B). Analysis of the Fab-E protein interface suggests that all complementarity determining regions (CDR) of the heavy and light chains are involved in the interaction (Fig. S6C).

Table 1: Fitting of DENV 1 E protein domains into HM14c10:DENV1 cryoEM density.

E protein in asymmetric unit[a]	E protein domain	Number of fitted atoms	Average map value at atom positions before[b] / after fitting[c]	Number of atoms outside contour before[b] / after fitting[c]	Shift from previous position (Å)	Rotation from previous position (°)
A	I	889	3.790 / 4.291	447 / 362	2.63	16.1
	II	1,260	4.635 / 5.212	533 / 424	2.16	3.02
	III	1,522	3.592 / 4.141	816 / 695	2.98	6.07
B	I	889	4.126 / 4.368	403 / 368	1.37	12.7
	II	1,260	4.715 / 5.359	539 / 409	2.30	4.51
	III	1,522	4.185 / 4.380	685 / 648	1.48	4.75
C	I	889	4.033 / 4.261	407 / 379	1.93	10.2
	II	1,260	4.744 / 5.098	499 / 428	1.06	7.37
	III	1,522	4.100 / 4.345	739 / 668	1.64	6.03

[a] For designation of E protein position see Fig. 12.

[b] Dengue 1 E protein domains were first superimposed onto the E protein positions of the cryoEM structure of mature dengue 2 virus (*27*).

[c] The fit of dengue 1 E protein domains into the HM14c10:DENV1 cryoEM map (set at a contour level of 4σ) were optimized by using the fit-in-map function in Chimera (*35*).

[00148] The binding footprints of the two HM14c10 Fabs in an asymmetric unit are not identical (Fig. 12D), with twelve amino acids common to both interfaces but four that are unique (Table 2). Sequence comparison of the epitope residues between different DENV1 isolates indicates that most residues are conserved (Fig. 20A), consistent with the observed neutralizing activity of HM14c10. In contrast, these residues are not conserved in other DENV serotypes or West Nile virus (WNV) (Fig. 20B).

Table 2. Fab HM14c10 epitope on DENV 1 E proteins.

Fab	E protein molecule in the asymmetric unit	E protein domain	E protein residues*
HM14c10(I)	A	I	**T51**, L135, K136, **G159**, **T160**, **T165**, **P166**, **Q167**, **E172**, **I173**, **T275**
	A	II	**N52**, **G274**
	B	III	**K310**, E384, K385
HM14c10(II)	B	I	**T51**, Q131, Y132, **G159**, **T160**,

		T165, P166, Q167, E172, I173,
		L175, **T275**
B	II	**N52, G274**
C	III	L308, **K310**

*Residues common in both epitopes bound by Fab HM14c10(I) and HM14c10(II) are indicated in bold.

Example 3: Time lapse confocal microscopy reveals the neutralization mechanism of HM14c10.

[00149] Antibodies can neutralize viral infections by diverse mechanisms including inhibition of virus attachment or fusion to endosomal membranes, or through blocking virally-induced conformational changes of the surface glycoproteins (*20, 21*). To understand the mechanism of HM14c10 neutralization of DENV1, time lapse confocal microscopy was employed to track the infection of cells by live, fluorescently tagged DENV (*22*) (Fig. 13 and 21A). When BHK cells were incubated with DENV1 and isotype control Mabs (non-DENV binding), the virus coalesced in multiple, predominantly perinuclear, intra-cellular compartments (Fig. 14A(a)). Neutralizing concentrations of HM4G2 induced the formation of viral aggregates in the extracellular space but these were also successfully internalized, confirming that HM4G2 does not inhibit virus attachment/internalization (Fig. 13A(b)). In contrast, HM14c10 induced the formation of smaller aggregates, but efficiently blocked attachment, with most of the small viral particles remaining in the extracellular space after one hour (Fig. 13A(c)). HM4G2 delayed the accumulation of intracellular viruses compared to the isotype control (Fig. 13B, upper and middle panels). HM14c10:DENV1 complexes failed to enter cells but could be seen deflecting from their surface (Fig. 13B, lower panel). The degree of fluorescent DENV1 internalized under all three conditions was quantified (Fig. 13C). These data suggest that the primary mode of inhibition of DENV1 by HM14c10 is through a blockage of virus attachment to host cells.

Example 4: HM14c10 exhibits great prophylactic and therapeutic activity *in vivo*.

[00150] DENV is not a natural pathogen in immunocompetent rodents, it is possible to induce a dose-dependent viremia in AG129 mice deficient in receptors for Type I/II IFN. We injected these mice with unmodified DENV1 subcutaneously (model I, Fig. 21B(a)) or intraperitoneally (model II, Fig. 21B(b)) then quantified viremia 3-4 days later respectively

(*20*). Two DENV1 clinical isolates, representing disparate genotypes (EHI-D1 genotype I versus Westpac genotype IV), were utilized to determine the *in vivo* efficacy of HM14c10. In both models, HM14c10 prevented disease when given to mice 24-hours before DENV1 infection, or when given 48 h after infection (Fig. 13D). The lowest concentration of HM14c10 where a significant reduction in viremia was observed is 0.6 μg per mouse (or 160 pM), representing an *in vivo* potency that has not been matched by any other reported anti-DENV therapeutic formulation.

Discussion

[00151] Recent reports on the humoral responses engendered by DENV infection (*23*, *24*) suggest that there is a dominance of antibodies that are mostly DENV serotype cross-reactive with weak neutralization activities. Although scarce in the human serum repertoire, E-DIII antibodies are suggested to protect against DENV infection (*23*, *24*) and this is consistent with studies on the murine antibody response to DENV (*7*). The Human antibodies characterized have principally been specific for DI and DII of the virus E protein. A small number of characterized antibodies were observed to bind to the whole virus but not to recombinant E protein suggesting specificity for quaternary structure dependent epitopes (*23*). In this study, we have isolated and thoroughly characterized a potent neutralizing antibody against DENV serotype 1. This antibody is highly neutralizing in both *in vitro* and *in vivo* systems. Since it binds only to DENV1, it does not cause enhanced infection of myelomonocytic cells by other DENV serotypes.

[00152] The 7Å resolution cryoEM structure of Fab HM14c10 complexed with DENV1, showed details of the binding between Fab and the E protein. This level of detail has not been observed in the previous cryoEM structures of antibody-Flavivirus complexes. The footprint of HM14c10 spans across E-DIII and E-DI:E-DII from a neighboring E protein (Fig. 12D). A report on a human antibody CR4354 specific for WNV has also implicated this region as a target for immunity (*25*). Although the cryoEM structure of WNV complexed with Fab CR4354 is solved to a lower resolution (14Å resolution) (Fig. 22A), the fitting of Fab CR4354 crystal structure generates a pseudo-atomic resolution structure. This allowed identification of interacting residues. Comparison of CR4354 epitope on WNV and HM14c10 epitope on DENV1 (Fig. 22B) showed that CR4354 has a bigger proportion of the footprint on E-DIII whereas HM14c10 has most of the interacting residues on E-DI. Sequence comparison of the epitopes showed that only approximately 20% of the CR4354 overlaps with HM14c10 epitopes, and the overlapping residues are mostly non-conserved (Fig. 22C).

[00153] Although CR4354 and HM14c10 epitopes are not identical, the binding of these antibodies should hold the neighboring E proteins together thereby locking the virus structure and preventing the conformation changes essential for a productive infection i.e., virus attachment to host receptors and fusion within the host cells endocytic pathway. Time-lapse live imaging confocal microscopy shows that HM14c10 inhibits the attachment of DENV to host cells. In contrast CR4354 was shown to preferentially inhibit WNV fusion suggesting that targeting this region by antibodies results in more than one mechanism of inhibition.

[00154] The surface proteins of DENV have been suggested to undergo constant changes at physiological condition – termed "breathing" (*21*). It is possible that breathing may play a role in facilitating attachment of the virus to cells. Since HM14c10 cross-links surface E proteins, it may then inhibit attachment by preventing the surface proteins from undergoing breathing. Alternatively, E-DIII has been shown to be important for host cell attachment, the binding of HM14c10 to E-DIII may thus sterically hinder this process. HMAb CR4354, although binding to similar region as HM14c10, does not inhibit WNV attachment. This implies that the encephalitis causing WNV and febrile illness causing DENV do not share identical receptor binding determinants.

[00155] HMAb CR4354 was shown to prevent fusion of virus to the endosomal membrane at low pH (*25*). Since HM14c10 also binds across neighboring E proteins, the possibility that HM14c10 may inhibit the rearrangement of dimeric E to trimeric structures during fusion cannot be ruled out. The potential to inhibit both receptor binding and fusion may explain the exceptional *in vivo* efficacy of HM14c10.

[00156] Most flaviviruses E-proteins have similar quaternary structure based on a high degree of similarity between the cryoEM structures of WNV (*26*) and DENV (*27*). Therefore, all flavivirus surface E proteins may undergo similar structural rearrangements during their infection cycle. Antibodies that target a similar region as HM14c10 or CR4354 in other flaviviruses may therefore be protective. Since HM14c10 and CR4354 antibodies are the only two antibodies characterized with this binding activity and both are derived from human sources, it indicates that this type of epitope is probably a determinant for generalized Flavivirus immunity. This has important implications for the design and evaluation of future vaccines.

[00157] Finally, given that HM14c10 has strong neutralization profiles against most clinical DENV1 isolates and excellent *in vivo* efficacy, this antibody represents a good therapeutic candidate for the treatment of DENV1 infected patients.

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[00158] While specific aspects of the invention have been described and illustrated, such aspects should be considered illustrative of the invention only and not as limiting the invention as construed in accordance with the accompanying claims.

[00159] All publications and patent applications cited in this specification are herein incorporated by reference in their entirety for all purposes as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference for all purposes.

[00160] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to one of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

CLAIMS

What is Claimed:

1. An isolated antibody or fragment thereof that binds specifically to a Dengue virus serotype 1 envelope (E) protein or fragment thereof, wherein the antibody is a human antibody with neutralizing activity that binds across two E proteins in a virus, wherein the binding across two E proteins comprises binding to DI and the hinge between DI and II on one E protein and DIII of a neighboring E protein,
wherein the antibody or fragment thereof comprises:
a heavy chain comprising three CDRs with the amino acid sequences SYGMH, VIWYDGSKTYYGDSVKG and GIAGGWAFW, and
a light chain comprising three CDRs with the amino acid sequences RASQNVYSYLG, GVTSRAT and QQYAG.

2. The antibody or fragment thereof according to claim 1, wherein the antibody or fragment thereof is selected from the group consisting of: (a) a whole immunoglobulin molecule; (b) an scFv; (c) a Fab fragment; (d) an F(ab')2; and (e) a disulfide linked Fv.

3. The antibody or fragment thereof according to claim 1 or 2, wherein the antibody or fragment thereof comprises:

a heavy chain immunoglobulin constant domain selected from the group consisting of: (a) a human IgM constant domain; (b) a human IgG1 constant domain; (c) a human IgG2 constant domain; (d) a human IgG3 constant domain; (e) a human IgG4 constant domain; and (f) a human IgA1/2 constant domain.

4. The antibody or fragment thereof according to claim 1 or 2, wherein the antibody or fragment thereof comprises:

a heavy chain with the amino acid sequence
EVQLVESGGGVVQPGRSLRLSCAASGFSFSSYGMHWVRQAPGKGLE
WVAVIWYDGSKTYYGDSVKGRFTISKDNSKKMVNLQMDSLGVEDTA
FYYCARGIAGGWAFWGIDLWGQGTLVTVSS.

5. The antibody or fragment thereof according to claim 1 or 2, wherein the antibody or fragment thereof comprises:

a heavy chain framework of IGHV1-2*02 and three of the CDRs with the amino acid sequences SYGMH, VIWYDGSKTYYGDSVKG and GIAGGWAFW.

6. The antibody or fragment thereof according to claim 1 or 2, wherein the antibody or fragment thereof comprises:

a light chain immunoglobulin constant domain selected from the group consisting of: (a) a human Ig kappa constant domain; and (b) a human Ig lambda constant domain.

7. The antibody or fragment thereof according to claim 1 or 2, wherein the antibody or fragment thereof comprises:

a light chain with the amino acid sequence

DVVMTQSPGTLSLSPGERATLSCRASQNVYSYLGWYQHKPGRSPRLLI
FGVTSRATGVPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYAGSAY
TFGQGTKVEIKR

or

DIVMTQSPGTLSLSPGERATLSCRASQNVYSYLGWYQHKPGRSPRLLIF
GVTSRATGVPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYAGSAYT
FGQGTKVEIKR.

8. The antibody or fragment thereof according to claim 1 or 2, wherein the antibody or fragment thereof comprises:

a light chain framework of IGKV3-20*01 and three of the CDRs with the amino acid sequences RASQNVYSYLG, GVTSRAT and QQYAG.

9. The antibody or fragment thereof according to any one of claims 1-8, wherein the antibody:

binds to a Dengue virus having the binding specificity of an antibody with the heavy chain amino acid sequence

EVQLVESGGGVVQPGRSLRLSCAASGFSFSSYGMHWVRQAPGKGLE
WVAVIWYDGSKTYYGDSVKGRFTISKDNSKKMVNLQMDSLGVEDTA
FYYCARGIAGGWAFWGIDLWGQGTLVTVSS and the light chain amino acid sequence
DVVMTQSPGTLSLSPGERATLSCRASQNVYSYLGWYQHKPGRSPRLLI
FGVTSRATGVPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYAGSAY
TFGQGTKVEIKR
or
DIVMTQSPGTLSLSPGERATLSCRASQNVYSYLGWYQHKPGRSPRLLIF
GVTSRATGVPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYAGSAYT
FGQGTKVEIKR.

10. The antibody or fragment thereof according to any one of claims 1-8, wherein the antibody:

is an antibody with the heavy chain amino acid sequence
EVQLVESGGGVVQPGRSLRLSCAASGFSFSSYGMHWVRQAPGKGLE
WVAVIWYDGSKTYYGDSVKGRFTISKDNSKKMVNLQMDSLGVEDTA
FYYCARGIAGGWAFWGIDLWGQGTLVTVSS and the light chain amino acid sequence
DVVMTQSPGTLSLSPGERATLSCRASQNVYSYLGWYQHKPGRSPRLLI
FGVTSRATGVPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYAGSAY
TFGQGTKVEIKR
or
DIVMTQSPGTLSLSPGERATLSCRASQNVYSYLGWYQHKPGRSPRLLIF
GVTSRATGVPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYAGSAYT
FGQGTKVEIKR.

11. The antibody or fragment thereof according to any one of claims 1-10, wherein the antibody or fragment thereof binds to an antigen with an affinity constant (K_D) of less than 1 x 10^{-8} M, and optionally binds to an antigen with an affinity constant (K_D) of less than 1 x 10^{-9} M.

12. The antibody or fragment thereof according to any one of claims 1-11, wherein the antibody is derived from a B cell of a patient who has recovered from Dengue virus infection.

13. A pharmaceutical composition comprising the antibody or fragment thereof according to any one of claims 1-12 and a pharmaceutically acceptable carrier effective to reduce or prevent Dengue virus infection in a subject.

14. The pharmaceutical composition of claim 13, further comprising a second agent.

15. An antibody or fragment thereof according to any one of claims 1-12 for use in a method of passive immunization against Dengue virus infection wherein an effective amount of the antibody or fragment thereof according to any one of claims 1-12 is to be administered to a subject.

16. An antibody or fragment thereof according to any one of claims 1-12 for use in a method of treatment of Dengue virus infection, wherein the antibody or fragment thereof according to any one of claims 1-12 is to be administered to a subject in need thereof in an amount that is effective to reduce or prevent the disease.

17. The antibody for the use according to claim 16, wherein:
the antibody is to be administered intravenously (IV), subcutaneously (SC), intramuscularly (IM), transdermally, or orally; or
the antibody is to be administered in an amount in the range of 1 to 100 milligrams per kilogram of the subject's body weight; or
said use further comprising a second agent to be administered to the subject, optionally wherein the second agent is antiviral drug or analgesic drug.

18. An isolated nucleic acid encoding the antibody or fragment thereof according to any one of claims 1-12.

19. An expression vector comprising the nucleic acid of claim 18.

20. A host cell comprising the expression vector of claim 19, optionally wherein the host cell is a bacterial cell, a eukaryotic cell, or a mammalian cell.