

**Generation and characterization of cross neutralizing human monoclonal antibody
against 4 serotypes of dengue virus without enhancing activity**

Subenya Injampa^{a,b}, Nataya Muenngern^b, Chonlatip Pipattanaboon^b, Surachet
Benjathummarak^b, Khwanchit Boonha^b, Hathairad Hananantachai^a, Waranya Wongwit^a,
Pongrama Ramasoota^{a,b}, Pannamthip Pitaksajjakul^{a,b,*}

^aDepartment of Social and Environmental Medicine, Faculty of Tropical Medicine, Mahidol
University, Thailand

^bCenter of Excellence for Antibody Research, Faculty of Tropical Medicine, Mahidol
University, Thailand

*Corresponding author at: Department of Social and Environmental Medicine, Faculty of
Tropical Medicine, Mahidol University, Bangkok, Thailand, 420/6 Ratchathewi, Bangkok,
Thailand, 10400

E-mail address: pannamthip.pit@mahidol.ac.th (P. Pitaksajjakul).

Abstract

Background. Dengue disease is a leading cause of illness and death in the tropics and subtropics. Most severe cases occur among patients secondarily infected with a different dengue virus (DENV) serotype compared with those with the first infection, resulting in antibody-dependent enhancement activity (ADE). Our previous study generated the neutralizing human monoclonal antibody, D23-1B3B9 (B3B9), targeting the 1st domain II of E protein, which showed strong neutralizing activity (NT) against all four DENV serotypes. However, at sub-neutralizing concentrations, it showed ADE activity *in vitro*.

Methods. In this study, we constructed a new expression plasmid using the existing IgG heavy chain plasmid as a template for Fc modification at position N297Q by site-directed mutagenesis. The resulting plasmid was then co-transfected with a light chain plasmid to produce full recombinant IgG (rIgG) in mammalian cells (N297Q-B3B9). This rIgG was characterized for neutralizing and enhancing activity by using different FcγR bearing cells. To produce sufficient quantities of B3B9 rIgG for further characterization, CHO-K1 cells stably secreting N297Q-B3B9 rIgG were then established.

Results. The generated N297Q-B3B9 rIgG which targeted **to** the conserved N-terminal fusion loop of DENV envelope protein showed the same cross-neutralizing activity to all four DENV serotypes as those of wild type rIgG. In both FcγRI- and RII-bearing THP-1 cells and FcγRII-bearing K562 cells, N297Q-B3B9 rIgG lacked ADE activity against all DENV serotypes at sub-neutralizing concentrations. Fortunately, the N297Q-B3B9 rIgG secreted from stable cells showed the same patterns of NT and ADE activities as those of the N297Q-B3B9 rIgG obtained from transient expression against DENV2. Thus, the CHO-K1 stably expressing N297Q-B3B9 HuMAb can be developed as high producer stable cells and used to produce sufficient amounts of antibody for further characterization as a promising dengue therapeutic candidate.

Discussion. Human monoclonal antibody, targeted to fusion loop of envelope domainII (EDII), was generated and showed cross-neutralizing activity to 4 serotypes of DENV, but did not cause any viral enhancement activity *in vitro*. This HuMAb could be further developed as therapeutic candidates.

Introduction

Dengue infection is a leading cause of illness and death in the tropics and subtropics. More than one-third of the world's population lives in areas at risk (Natasha Evelyn and Mikkel, 2013). DENV is a member of the *Flavivirus* genus in the *Flaviviridae* family. There are four antigenically different serotypes of DENV (DENV-1 to DENV-4) (Kuhn et al., 2002). Primary infection with one DENV serotype induces the production of homotypic neutralizing antibodies, which provide lifelong immunity against the infecting serotype. These homotypic neutralizing antibodies can cross-react but less specific against the other three DENV serotypes and persist for a period of several months to a few years (Guzman MG and Vazquez S, 2010). Upon secondary infection with heterologous DENV serotypes, these antibodies fail to neutralize virus resulting in an increase of infected cells and higher virus titers. This phenomenon is named antibody-dependent enhancement activity (ADE) (Halstead SB and O'Rourke EJ, 1977). Primary infection typically causes asymptomatic or self-limited dengue fever (DF). Whereas patients in secondary infection usually have an increased risk of developing severe disease, including life-threatening dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS)(Murphy BR and Whitehead SS, 2011).

Currently, there is no available antiviral drug specific for all four DENV serotypes. Even though there are many studies of neutralizing antibodies against DENV, fully human monoclonal antibodies (HuMAbs) which are able to neutralize all four DENV serotypes without ADE activity are considered to be the main option for passive immune therapy (Marasco WA and Sui J, 2007; Chan AC and Carter P, 2010; Low et al., 2017).

Previously, we generated HuMAb clone D23-1B3B9 (B3B9) with strong *in vitro* neutralizing activity (NT) against DENV-1 to DENV-4 (Setthapramote et al., 2012). However, investigating the viral infection enhancing activity of this HuMAb on Fc-gamma receptor (FcγR)-bearing cells revealed an increase in DENV infection at sub-neutralizing

concentrations, which limits its application as a therapeutic candidate (Sasaki et al., 2013). To diminish enhancing activity, modifications at glycosylation site Asn297, which influence the binding affinity between Fc region and FcγR presenting on immune effector cells, were established (Hristodorov et al., 2013; Chan AC and Carter PJ, 2010; Balsitis et al., 2010). Glycosylation is a key player in IgG –mediated immunity, antigen recognition of invading microorganisms and the interaction with FcγRs that lead to cellular and effector activities (Hayes et al., 2014; Jefferis et al., 1998). Interaction with the FcγR on innate immune cells allows the IgG molecule to communicate with the immune system and induce immune effector functions such as antibody dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) (Kellner et al., 2014), and phagocytosis following opsonization of target antigens (Algirdas et al., 2015). Changing of glycosylation pattern influences antibody-mediated effector functions (Nimmerjahn et al., 2007). Studies of ligand binding and IgG-induced cellular responses have demonstrated that Fc gamma receptor recognition is impaired for aglycosylated forms of IgG (Sazinsky et al., 2008; Nesspor et al., 2012).

We constructed plasmids expressing antibody light chain (LC) and modified the Fc region of the heavy chain (HC) constant domain 2 at position N297Q by site-directed mutagenesis. The modified HC plasmid was co-transfected with the LC plasmid into HEK293T mammalian cells to produce full recombinant IgG (rIgG). To evaluate the Fc-modified rIgG as a potential therapeutic candidate for dengue treatment, this Fc-modified rIgG was characterized and determined its NT and ADE activity *in vitro* and compared with those of wild type rIgG. We also demonstrated the mimic binding epitope on envelope protein of dengue virus for understanding specificity and functionality of this HuMAb.

Materials and Methods

Cell lines and DENV strains

HEK293T cells were maintained in Dulbecco's modified Eagle medium (Gibco, Grand Island, New York, USA.) with 10% fetal bovine serum (FBS). For NT test, Vero cells were cultured in minimal essential medium (MEM) (GE Healthcare UK Ltd., Buckinghamshire, UK) with 10% fetal bovine serum. THP-1 and K562 cells, which were used in ADE assays, were cultured in RPMI 1640 medium (Gibco) with 10% FBS. The DENV strains used in this study were the Mochizuki strain of DENV1, the 16681 strain of DENV2, the H87 strain of DENV3, and the H241 strain of DENV4. All DENVs were propagated in C6/36 cells, which were maintained in Leibovitz's L-15 medium (Gibco) supplemented with 10% FBS and 0.3% of BACTO Tryptose Phosphate Broth (TPB) (Sigma-Aldrich, Missouri, USA). CHO-K1 cells were maintained in MEM medium supplemented with 10% FBS and 1% non-essential amino acid (Gibco). All cell lines were kindly provided by Research Institute for Microbial Diseases, Osaka University.

Generation of wild type and mutated human monoclonal antibody clone B3B9

Plasmid construction

Variable HC and LC sequences of B3B9 HuMAb were previously isolated from hybridoma cells (Pitaksajjakul et al., 2014). Wild type HC and LC expression plasmids containing variable and constant regions were previously constructed (Pitaksajjakul et al., 2014). In this study, HC plasmid of **wild type was** used as a template for site-directed mutagenesis with the In-Fusion Cloning System (In-Fusion® HD Cloning Plus; Clontech Laboratories Inc., Shiga, Japan). Primers were designed according to the manufacturer's instructions to mutate the amino acid at position 297 from asparagine to glutamine. This system combines the action of the In-Fusion HD enzyme with inverse polymerase chain reaction (PCR), which generates linearized DNA from a plasmid template. The PCR reaction

was composed of the CloneAmp Hifi PCR premix, 300 nM each of forward and reverse primer, 5 ng of plasmid, and distilled water to final volume of 25 µl. The amplification was performed with 35 cycles of 98 °C for 10 seconds, 55 °C for 15 seconds, and 72 °C for 5 seconds. The inverse PCR products were gel-purified using a PureLink® Quick Gel Extraction Kit (Invitrogen, California, USA.) following the manufacturer's protocol. The linearized plasmids obtained from inverse PCR were then fused by the In-Fusion enzyme following the manufacturer's instructions. The reaction was performed at 50 °C for 15 minutes. The in-fusion reaction was chemically transformed into Stella chemical competent cells supplied by the kit. Individual clones were randomly selected for DNA sequencing.

DNA sequencing and plasmid preparation

The DNA sequencing of the mutated HC plasmids were confirmed by Sanger sequencing (Macrogen Inc., Korea). Potential N-glycosylation site of wild type and mutated B3B9 HuMAb was identified using NetNGlyc 1.0 software (Gupta et al., 2004). Plasmids that contained the target mutation were amplified in *E. coli* and isolated using a Purelink™ plasmid Midiprep kit (Invitrogen) from 100 ml culture for further transfection in mammalian cells.

Transient expression of N297Q-B3B9 rIgG in HEK293T cells

Plasmids expressing N297Q-B3B9 HC and LC were transfected to HEK293T cells to produce whole rIgG with the N297Q mutation as previously described (Pitaksajjakul et al., 2014). The culture medium containing secreted N297Q-B3B9 rIgG was collected and used in immunofluorescence assays (IFAs) to determine the DENV-binding activity. The N297Q-B3B9 rIgG was purified using a protein A affinity column and measured the concentration of N297Q-B3B9 rIgG by BCA protein assay kit (Thermo Scientific, USA.). The purity of

purified antibody was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

IFA for DENV-binding activity

IFAs were used to determine the binding and specificity of N297Q-B3B9 rIgG for all four DENV serotypes. IFA plates were prepared by infecting Vero cells with DENV at a multiplicity of infection of 0.01 as well as mocked infected. The plates were incubated for 3 days before being fixed and permeabilized with 3.7% paraformaldehyde and 0.1% Triton X-100, respectively. Culture fluid from transfected cells was added, and the plates were incubated at 37 °C for 1 h. AlexaFluor 488-conjugated anti-human IgG (1:1000 dilution) (Invitrogen) was added as secondary antibody. The result was observed under fluorescence microscope (IX71, Olympus). For this assay, non-infected cells were used as a negative control.

HuMAb isotyping

The IgG isotype of the N297Q-B3B9 rIgG was determined by PCR as previously described (Omokoko et al., 2014) using complementary DNA isolated from HuMAb clone B3B9 hybridoma as a template. The gene-specific primers used to amplify IgG1, -2, -3, and -4 are listed in Supplementary Table 1. IgG1 and IgG3 were amplified using the same forward and reverse primers. The primers were used to amplify at the hinge region between the Fab and Fc parts that produce different sized PCR products. IgG1 and IgG3 showed specific bands at 211 and 346 bp, respectively. With separate PCR reactions, IgG2 and IgG4 were amplified by the same reverse and different forward primers. These IgG2 and IgG4 PCR reactions showed 207 bp and 210 bp, respectively. The PCR reaction was composed of 0.5 µg of cDNA, 1 mM Tris-HCl (pH 8.0), 5 mM KCl, 1.25 mM deoxyribonucleotide triphosphate

(dNTP), 1.25 units of ExTaq DNA polymerase (TAKARA, Shiga, Japan), and 5 mM of each primer, with distilled water to a final volume of 25 µl. The amplification of each primer pairs was performed in separate reaction with 35 cycles of 94 °C for 30 seconds, 65 °C for 30 seconds, and 72 °C for 30 seconds. The PCR products were separated by agarose gel electrophoresis and visualized by staining with SYBR Safe DNA Gel stain (Invitrogen).

Foci reduction neutralization test (FRNT)

FRNT assay was performed as previously described (Pitaksajjakul et al., 2014). DENV with MOI 0.01 were mixed with different concentrations of purified HuMAbs (B3B9 or N297Q-B3B9 rIgG) and incubated at 37 °C for 1 hour. Each mixture was added to Vero cells in 96-well cell culture plates and incubated for 2 hours. The plates were then overlaid with 2% carboxymethyl cellulose (CMC) in MEM medium with 2% FBS and incubated for 2 days for DENV4 and for 3 days for DENV1, 2, and 3. After that, the plates were fixed with 3.7% paraformaldehyde/PBS and 0.1% Triton X-100/PBS. Immunostaining was performed by an incubation with anti-DENV human antibodies, followed by Alexa-conjugated anti-human IgG (H+L) (1:1000 dilution). Foci numbers were counted under a fluorescence microscope. The percent reduction was calculated by comparing the foci number for each antibody concentration with the number of foci obtained from a virus–PBS mixture (negative control).

Antibody dependent enhancement (ADE) assay on K562 cells

The ADE activity of the N297Q-B3B9 rIgG was also assessed on FcγRIIa-bearing K562 cells (Konishi et al, 2010). Antibodies at serial four-fold dilutions and viruses were mixed in 10% FBS RPMI medium in 96-well poly-L-lysine-coated plates (Corning Inc., New York, USA) and incubated at 37 °C. After 2 hours, 50 µl of 2×10^6 cells/ml K562 cells were

added. The cell–HuMAb–virus mixture was co-cultured at 37 °C under 5% CO₂ for 2 days. The cells were then fixed with an acetone/methanol fixing solution at –20 °C. Immunostaining was performed by adding an anti-DENV monoclonal antibody and incubating at 4 °C overnight. Horseradish peroxidase-conjugated anti-human IgG (H+L) diluted in 0.05% Tween and 1% FBS in PBS was then added, and the samples were incubated at 37 °C for 1 hour. The signal was developed with a DAB substrate solution (KPL, Maryland, USA). Infected cell counts obtained from the test were adjusted with the mean infected cell counts obtained with the four negative controls set in the same experiment. The cut-off value for differentiating enhancing from non-enhancing activities was calculated from the average plus three times the SD of the percentages of infected cells obtained with the four negative controls.

Antibody dependent enhancement (ADE) assay on THP-1 cells

DENV at a multiplicity of infection of 0.1 was incubated with serially diluted antibodies in serum-free RPMI medium at 37 °C for 1 hour. Then, 150 µl of 5×10⁵ cells/ml THP-1 cells, which express both FcγRI and II on their surfaces, were added and the samples were incubated at 37 °C under 5% CO₂. After 2 hours, RPMI medium with 4% FBS was added. The cell–HuMAb–DENV solution was incubated for 3 days, after which RNA was extracted from the infected cells using TRIzol[®] reagent (Invitrogen). The viral RNA was quantified by a one-step Real time PCR using dengue virus specific primer and glyceraldehyde 3-phosphate dehydrogenase (GADPH) as an internal control (Sasaki et al., 2013). The result is presented as the fold enhancement of virus copies compared with the virus–PBS mixture (control).

Epitope mapping by phage display of random peptide libraries

Phage affinity selection (Biopanning)

Human monoclonal antibody clone B3B9 that show cross-neutralizing activity to 4 serotypes of DENV was used for mapping the epitope (Rowley et al., 2004). Panning was performed by using Ph.D.-12 and Ph.D.-C7C Phage Display Peptide Library Kit (New England Bio Labs Inc., UK) following the **manufacture protocol**. As described in the manufacturer lab manual, the dodeca- and loop-constrained hepta- peptide library consists of 1.2×10^9 electroporated sequences.

Briefly, 50 μ l of protein A/G magnetic beads were blocked with BSA by incubating at room temperature for 1 hour, and washed with 0.1% Tris Buffered Saline with Tween (TBST). In the meantime, Phages (5×10^{10} plaque forming units (PFU)) were incubated at room temperature for 30 minutes with purified B3B9 HuMAb to a final volume of 200 μ l TBST. Phage - HuMAb mixture was transferred to the tube containing the blocked magnetic beads and incubated for 20 minutes at room temperature. After incubation, nonbinding phages that were not captured on magnetic beads were washed with TBST. The bound phages were eluted with 1 ml glycine elution buffer (0.2 M Glycine-HCl, 1 mg/ml BSA, pH 2.2) and neutralized with 150 μ l of 1M Tris-HCl (pH 9.1). Then, the bound phages were directly added to 20 ml early-log Escherichia coli (E. coli) ER2738 culture and incubated at 37°C with vigorous shaking **for** 4.5 hours. After incubation, the culture was transferred to a 50 ml centrifuge tube and spun at 5,000 rpm for 15 minutes at 4 °C. The phage-containing supernatant was transferred to a new 50 ml centrifuge tube. These amplified phages were precipitated with 20% PEG 8000/2.5 M NaCl at 4 °C **for** overnight, and centrifuged at 12,000 g for 15 minutes. Phage pellets were then resuspended with 200 μ l TBS and used in the next cycle. Three rounds of selection were performed. Specificity of selected phage clones were confirmed by phage ELISA. Briefly, 96-well microtiter plates were coated in triplicate well of 100 μ l B3B9 HuMAb at 4°C overnight. Then, the wells were washed 2 times with 0.05%

Phosphate Buffered Saline with Tween (PBST). After that, the wells were blocked with blocking buffer (3% BSA in TBST). Then, phage lysate of each selected clones from panning step was added to 2 wells of coated antibody. At this step, wild type M13 phages were added to another HuMAb-coated well as a no fusion peptide control. Consequently, anti-M13 antibody-HRP conjugated; dilute 1:5000 in blocking buffer were added. All incubations were performed in humidity chamber at 37°C, for 1 hour. After washing, the reaction was developed using 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (Sigma-Aldrich), and terminated by adding 100 µl of 2.0 M H₂SO₄. The absorbance was measured at 450 nm using ELISA reader (TECAN). Plasmids from positive clones of ELISA were isolated and sequenced using -96 gIII sequencing primer 5'-TGA GCG GAT AAC AAT TTC AC-3'. This primer was used to amplify the fusion peptide region. The inserted random 7 and 12 amino acids were identified and analyzed. All inserted sequences were aligned to determine the consensus sequence. The matched peptides of consensus sequences with dengue viral genome were analyzed by sequence alignment.

Phage affinity binding by inhibition ELISA

After sequence analysis, inhibition ELISA was used to confirm candidate phages from each group of inserted peptide sequences that had specifically bound to B3B9 HuMAb. Reciprocal dilution of each phage clones were firstly determined to use in inhibition assay. For inhibition ELISA, B3B9 HuMAb was serially 2-fold diluted started at 20 µg/ml. Then, the diluted antibody was incubated with equal volume of phage at specified dilution at room temperature for 1 hour. After incubation, the mixtures were transferred to HuMAb pre-coated plate, and incubated for another 1 hour. Bound phages to the coated HuMAb were detected by anti-M13-HRP, and signal was developed with TMB substrate. The absorbance values were measured at 450 nm using an ELISA reader (TECAN). Absorbance values of each

concentration (A) of antibody in solution phase were divided by absorbance values in the absence of antibody (A0), yielding normalized values (A/A0) (Skottrup et al., 2012). Non-relevant anti-influenza antibody, which was tested as negative binding with our selected dengue-specific phage clones, was used as a negative control.

Generation of stable, antibody-secreting CHO-K1 cells

The HC-expressing constructed plasmid (pQCXIP-CH) contained a puromycin-resistant gene and the LC-expressing constructed plasmid (pQCXIH-CL) contained a hygromycin-resistant gene. After determining the optimal concentrations of the corresponding two antibiotics, stable antibody-secreting CHO-K1 cells were selected using puromycin and hygromycin at 8 µg/ml and 800 µg/ml, respectively. For stable cell generation, briefly, CHO-K1 cells were seeded in a 6-well cell culture plate the day before transfection to obtain a cell density of 90–95% on the next day. The medium was replaced with Opti-MEM I reduced serum medium. Plasmid DNA was mixed with transfection reagent (Lipofectamine 2000) and added to the cells, which were then incubated for 5 hours. After that, the medium was replaced with culture medium (10% FBS, 1% NEAA MEM). The cells were incubated at 37 °C with 5% CO₂ for 24 hours. After that, the medium was replaced with culture medium containing the two antibiotics at the concentrations specified above. The medium was changed every 3–4 days until 60% of alive cells were observed. The transfected cells were used for cell cloning by limiting dilution on 96 well-cell culture plates and incubated for 10–14 days with selection media. The positive clones that were able to secrete the target anti-DENV rIgG were selected by IFA using the culture supernatant of each well containing a single stable clone. The cells were scaled up for further characterization. The level of IgG contained in the culture fluid of each positive clone was roughly determined by an IgG quantitation enzyme-linked immunosorbent assay (Bethyl Laboratories, Inc., Texas, USA.).

The functionalities of the purified N297Q rIgG secreted from the stable cells were confirmed by IFAs, NT and ADE assays.

Results

Aglycosylated HuMAb clone B3B9 expressed by transient expression system showed cross neutralizing activity to all serotypes of DENV without ADE activity

Wild type and mutated human monoclonal antibody clone B3B9 were generated and showed cross reactivity to all serotypes of DENV

The substitution of amino acid at position 297 from Asparagine to glutamine was created and confirmed by DNA sequencing. Five mg of wild type B3B9 and four mg of N297Q-B3B9 HuMAb were obtained. The successful production of wild type and mutant (N297Q) rIgG was verified by the capacity of binding activity observed via IFA. The N297Q-B3B9 rIgG displayed cross-reactivity to all four DENV serotypes similarly to the wild type rIgG (Fig. 1A-D).

Human monoclonal antibody clone B3B9 is IgG1 isotype

Reverse transcription PCR was performed to determine the B3B9 rIgG isotype. Using B3B9 hybridoma complementary DNA as template, the PCR product with approximately 211 bp of IgG1 was observed (Supplement fig. 1).

Wild type and mutated human monoclonal antibody clone B3B9 showed cross neutralizing activity against all serotypes of DENV

To determine the neutralizing activity of rIgG, we assessed the NT of N297Q-B3B9 rIgG and B3B9 rIgG in Vero cells. N297Q-B3B9 rIgG displayed almost the same level of NT

as the B3B9 rIgG for (change to _against_) all DENV serotypes. The NT levels of various concentrations of these two antibodies against all four DENV serotypes are shown in Fig. 2A-D. Among these four serotypes, B3B9 and N297Q-B3B9 rIgG showed identical 50% reduction of FFU (NT50) to DENV-2, 3, and 4 (0.125 µg/ml for DENV2 and 2 µg/ml for both DENV3 and DENV4). However, the NT50 concentration against DENV1 of N297Q-B3B9 rIgG was slightly higher than B3B9 rIgG (Fig.2E).

Mutated human monoclonal antibody clone B3B9 eliminated ADE activity in K562 cells while wild type showed ADE activity in (change to _against_) all types of DENV B3B9 rIgG was able to neutralize DENV2 and 3 at the minimum concentration, 0.39 and 25 µg/ml respectively (FIG????). Different from DENV1 and 4 which B3B9 rIgG did not show neutralizing activity(????). Moreover, this antibody induced a virus infection enhancement at concentrations 400-0.0015 µg/ml, 0.39–0.006 µg/ml, 100–0.0015 µg/ml, and 400-0.00038 µg/ml for DENV1, 2, 3, and 4, respectively (Fig. 3A-D). In contrary, N297Q-B3B9 rIgG showed NT for all four DENV serotypes and showed no enhancing activity in any of the tested antibody concentrations.

Mutated human monoclonal antibody clone B3B9 showed no ADE activity in THP-1 cells while wild type showed ADE activity in all types of DENV

In THP-1 cells, mutant IgG (N297Q-B3B9) that cannot bind to FcγR showed a complete reduction of ADE activity in all tested antibody concentrations. In contrast, wild type rIgG induced 9541, 43, 3061, and 2020 fold enhancement in the dengue virus copy (genomes?) number compared with the control for DENV1, 2, 3, and 4, respectively (Fig. 3E-H).

Epitope of B3B9 rIgG was within the highly conserved N-terminal fusion loop peptide of the E protein domain II

Biopanning of a phage display C7C and 12 random peptide library was performed using the affinity selection of purified B3B9 HuMAb. After biopanning, the number of output phages obtained from Ph.D. C7C and Ph.D.12 increased from 6.9×10^5 to 9.8×10^8 PFU and 6.9×10^5 to 7.7×10^8 PFU respectively, which proposed that specific enrichment had occurred (Fig 4A). Plasmids of phage clones that show binding activity with HuMAb by ELISA were isolated for DNA sequencing. Inserted oligonucleotide sequences of phage DNAs were translated to peptide sequences. Peptide sequences were aligned using BioEdit program 7.2.3 to analyze the epitopes and the binding motif of HuMAb. Phage clones were shown to display a consensus sequence LXXXG (Fig 4B). After comparing this peptide with the amino acid sequences of E proteins of DENV1–4, it was found that this LXXXG motif matched to 107LFGKG111 within the highly conserved N-terminal fusion loop peptide of the E protein domain II (Fig 4C). To investigate the solution-phase binding of each candidate phage random peptide with target B3B9 HuMAb, eight candidates phage clones which matched to the motif of 107LXXXG111 were selected for this study (Fig 4B, Table 1). From phage inhibition ELISA, it was found that the binding of all phage clones were inhibited by HuMAb (which one? As a reader, no one has to guess????) with dose-dependent manner, as shown in Fig 4D.

N297Q-B3B9 rIgG producing from CHO-K1 stable cell lines showed neutralizing activity against DENV2 without enhancing activity

From 125 stable clones that were screened, one showed the highest IgG secretion level (9,587 µg/ml). This clone was continually cultured to collect supernatant for purification. Preliminary study against DENV2 showed that all tested concentrations of the modified rIgG secreted from the stable cell line showed the result of viral NT (Fig. 5A) and no ADE activity (Fig. 5B), the result as modified rIgG secreted from transient expression.

Discussion

Due to the complexity of DENV infection and pathogenesis (Marasco and Sui, 2007), the ideal therapeutic antibodies should be fully human-derived and capable of inhibiting all four serotypes to reduce the risk of ADE causing more severe symptoms (Chan et al., 2013). We previously generated a cross-neutralizing HuMAb B3B9 (Setthapramote et al., 2012). Although this HuMAb showed strong NT to all DENV serotypes, at sub-neutralizing concentrations, it promoted ADE activity (Sasaki et al., 2013). To overcome this problem, we generated rIgG with an engineered Fc to prohibit the binding between Fc portion and Fc receptor.

The important structure (use another term...receptor? Molecule? Player?) for ADE mechanism is FcγR (Fc gamma receptor) that stimulates virus uptake and infection (explain that _after binding an immune complex_). Fc region (of what?) contains asparagine residue (N297) which is a single N-linked glycosylation site in its CH2 domain. The nature of this glycan can decisively influence the therapeutic performance of a recombinant antibody, and their absence or modification can lead to changing of conformation and losing Fc effector functions (Hristodorov et al., 2013; Subedi and Barb, 2015). These activities influence from the attaching sugar moiety at position N297 (not a clear sentence). Thus, deletion of the N-glycan changes the structure of the Fc portion resulting in diminished binding to Fcγ receptor (Nimmerjahn and Ravetch, 2008).

417 Firstly, we evaluated the generated N297Q-B3B9 rIgG for its neutralizing activity in
418 Vero cells and found that the engineering antibody showed proper neutralizing activity
419 comparable to B3B9 rIgG, except DENV1, with lower NT activity of N297Q-B3B9
420 comparing with B3B9 rIgG. This variation of NT50 concentration between B3B9 and
421 N297Q-B3B9 rIgG may be caused by low efficiency of B3B9 rIgG to neutralize DENV1
422 (Sasaki et al., 2013) Please elaborate, still no clear why?.

423 For K562 cells that express FcγR, these cells can assesses NT and ADE activity (not
424 clear written) (Konishi et al., 2010). In this simplified ADE assay, the virus infection
425 enhancement is represented as the number of infected cells from each antibody concentration
426 compared with those from a control performed in the absence of antibody. In this cell type,
427 we found that B3B9 rIgG, which showed cross-neutralizing activity to 4 DENV serotypes in
428 Vero cell, only neutralized DENV 2 and 3, but not DENV1 and 4 (FIG???). In contrary,
429 N297Q-B3B9 rIgG showed cross-neutralizing activity to 4 serotypes of DENV.

430 However, since K562 cell only expresses FcγRIIa, we further determined the ADE
431 activity in THP-1 cells that expressed both FcγRI and FcγRII. In accordance with ADE study
432 in K562 cells, our N297Q-B3B9 rIgG showed clearly reduction of viral enhancement activity
433 in THP-1 cell. This result showed the potential of N297Q-B3B9 rIgG as a human monoclonal
434 therapeutic antibody that cross neutralizes all serotypes of DENV without enhancing activity.

435 Many studies of Fc modification were explored for establishment of therapeutic
436 antibodies candidates against dengue virus. In 1989, Tao and Morrison studied roles of
437 aglycosylated chimeric mouse-human IgG antibody by modifying at glycan positions N297Q,
438 N297H and N297K via site directed mutagenesis (Tao and Morrison, 1989). These
439 aglycosylated antibodies cannot bind to human FcγRI and not trigger C1q binding ability of
440 complement system (Tao and Morrison, 1989). Balsitis et al. (2010) reported that a N297Q

441 mutation of mouse and chimeric human-mouse IgG that efficiency reduced ADE *in vitro*
442 could decreased the mortality of DENV-infected mice (Balsitis et al., 2010).

443 The results of this study differ to the one carried out by Ramadhany et al., 2016,
444 which modified human monoclonal antibody as (at instead of as???) N297A and showed NT
445 activity to be the same as its parental HuMAb. Nonetheless, this HuMAb still induced low
446 levels of virus infection enhancement (Ramadhany et al., 2015). One possible reason might
447 be the type of mutated amino acid used. Considering the amino acid structure, the substitution
448 of Asparagine with Glutamine (conservative mutation) reduces the effects of functional
449 properties because the side chains of these two amino acids differ by only one methylene
450 group. Thus the substitution amino acid is one of the essential factors that affect to the
451 efficiency of antibody.

452 Several studies have been approved for successful utilization of random peptide phage
453 display for finding specific epitope to several antiviral (Xue et al., 2012; Zhao et al., 2012),
454 and anti-flavivirus monoclonal antibody (MAb) (Sun et al., 2011). This technique provides an
455 economical and rapid approach for mapping map B-cell epitopes (Zhang et al., 2006; Chin et
456 al., 2017). We mapped the epitopes of our HuMAb to LXXXG which correspond to
457 107LFGKG111 located in the conserved N-terminal fusion loop of envelope domain II
458 (EDII). In accordance with our previous studies, this cross-neutralizing HuMAb B3B9 also
459 targeted to DII of envelope proteins residue 52-132, analyzed by western blot using truncated
460 E protein (Sasaki et al., 2013). This is a major target epitope of human antibodies for NT and
461 ADE activity (Costin et al., 2013; Deng et al., 2011). Comparing (TO WHAT???) with
462 epitopes targeted for both murine and human MAb specific to dengue virus (Shrestha et al.,
463 2010; Sukupolvi Petty S et al., 2010, Schieffelin et al., 2010; Beltramello et al., 2010; de
464 Alwis et al., 2012). It was found that MAbs generated from mice are mostly serotype-specific
465 that targeting DIII of envelope protein (Shrestha et al., 2010). However, most of the HuMAbs

were targeted to DI-II of envelope proteins which is more cross-reactive (Beltramello et al., 2010; de Alwis et al., 2012).

As a promising therapeutic candidate, it was described by Williams et al., (2013) that the chimeric N297Q MAbs targeting fusion loop and dimer interface on EDII, but not the A strand and C-C' loop on EDIII, acted therapeutically by competing against enhancing antibodies in polyvalent serum that recognize the same or proximal epitopes. This kind of fusion loop specific N297Q MAb showed protective activity *in vivo* when administered with enhancing titer of polyserum (Williams et al., 2013). Thus, the identification of B cell epitope in this study is crucial to understand its function and antibody/epitope interaction.

For antibody production, in this study, we used HEK293T cell for transient expression mainly because of its higher transfection efficiency and expression level, resulting in lower development costs when compare to stable cell line development (Zhang et al., 2012). However, for further characterization of N297Q-B3B9 rIgG as a dengue therapeutic candidate large quantities of antibody, with high stability in both production yield and quality, were required. Therefore, stable expression was used as an alternative solution for antibody production. CHO-K1 cells were used for stable cell line generation. This cell line is the prominent system for bio-manufacturing of therapeutic products (Hossler et al., 2009) as 70% of the therapeutic protein was produced by this system (Croset et al., 2012). Characterization of this antibody **in the other aspects (which ones???)** was required because protein produced by HEK293T cells and CHO-K1 cells show different patterns of glycosylation. However, we found no differentiation of NT and ADE activity in both cell types (Figure 5).

Together, our results suggest that N297Q-B3B9 rIgG is a human monoclonal antibody that can neutralize all four serotypes of DENV without viral enhancing activity. As a fully human-derived monoclonal antibody, it avoids the problem of a human anti-mouse antibody

response and after a thorough characterization, this N297Q B3B9 rIgG should be considered as an option for dengue therapeutic treatment.

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Figure Legend

Figure 1. Immunofluorescence assay of B3B9 and N297Q-B3B9 rIgG against four DENV serotypes. Vero cells were infected with DENV1, 2, 3, or 4 with MOI 0.1. The ability of the HuMAbs B3B9 (A-D) and N297Q-B3B9 rIgG (F-I) in the culture fluids (change for supernatants) of HEK293T cells transiently expressing these antibodies to bind to different serotypes of DENV was assessed by performing IFAs. (E,J) Negative control of mock infected cells. Representative images are shown.

Figure 2. Neutralizing activity of N297Q-B3B9 and B3B9 rIgG antibody against four DENV serotypes. NT levels of N297Q-B3B9 rIgG and B3B9 rIgG of DENV1-4 (A-D),in Vero cells were assessed by foci reduction neutralization tests. The foci of infected cells were counted and compared with the no antibody control, and the results were calculated as the percent reduction in focus forming units. The number of foci was calculated as the average of triplicate experiments. (The error bars show standard deviation of the experiments). (E) NT50 concentration (concentration that used for reduce half of infected cells when compared with control) of N297Q-B3B9 and B3B9 rIgG antibody against DENV serotypes.

Figure 3. ADE assays in K562 and THP-1 cells. (A-D) Enhancement activity against the four DENV serotypes in FcγRII-bearing K562 cells. The number of infected cells (log₁₀) from each antibody concentration compared with that of the control without antibody is shown. The numbers of infected cells were derived from the average of the counted infected cells from

three frames at 20x magnification multiplied by a surface area amplification factor to obtain the total number of cells in each well. Dotted lines indicate cut-off values for differentiating neutralizing and enhancing activity from the average plus three times the SD of the percentages of infected cells obtained with the four negative controls. (The error bars show standard deviation of the repeated experiments). (E-H) ADE assay on THP-1 cells. The antibody concentration was serially diluted ten-fold and are represented on the X-axis. The fold enhancement in the virus copy number of the sample with each antibody was compared with that of the no antibody control and is represented on the Y-axis. The plotted values were obtained from the average of duplicates from two repeated experiments.

Figure 4. Epitope mapping by phage display of random peptide libraries. (A) Affinity selection of phage-display. Ph.D.-12 and Ph.D.-C7C Phage Display Peptide Libraries were used in biopanning step. The constant units of phage (5×10^{10} PFU) was used for three rounds of biopanning. Increasing of percent yields of output phages represents the specific enrichment. (B) Alignment of phage-displayed peptide sequences selected by HuMAbs. Phage clones were shown to display a consensus sequences LXXXG (show in the box) (C) Comparison of the amino acid sequences of E proteins DENV1–4. In the box, DENV1–4 shared the same amino acids at positions 107 (L), 108 (F), 109 (G), 110 (K), and 111 (G). (D) Phage inhibition ELISA of 8 phage clones which matched to the motif of 107LXXXG111. Phage lysate of selected clones bound with HuMAb in solution phase and the free phage clones were detected by ELISA. The absorbance values were measured at 450 nm using an ELISA reader (TECAN). Absorbance values of each concentration (A) were divided by absorbance values of no antibody (control) (A0), resulting in normalized values (A/A0). Anti-influenza antibody was used as negative control.

Figure 5. The NT and ADE activity against DENV2 of N297Q-B3B9 rIgG derived from a stable and transient CHO-K1 cell line. A line of CHO-K1 cells stably expressing N297Q-

B3B9 rIgG was established. (A) The NT of N297Q-B3B9 rIgG against DENV2. The X-axis represents the concentration of antibodies, and the Y-axis represents the percent reduction in focus-forming units compared with the foci number of the control sample that lacked antibody. The number of foci was calculated as the average of triplicate experiments. (B) An ADE assay of N297Q-B3B9 rIgG against DENV2 was performed in K562 cells. The infected cells were counted at each antibody concentration and compared with the control (DENV2-infected cells without antibody). Dotted lines indicate cut-off values for differentiating neutralizing and enhancing activity from the average plus three times the SD of the percentages of infected cells obtained with the four negative controls. (The error bars show standard deviation of the repeated experiments).

Table 1. The consensus peptide sequences of eight selected phage clones. These clones matched to the motif 107LXXXG111 of DENV genome.

Supplement figure 1. PCR isotyping of N297Q-1B3B9 rIgG. First lane was 100 bps DNA ladder. Second lane was the sample that amplified from the same forward primer of IgG1 and IgG3; third lane was the sample amplified by forward primer of IgG2 and last lane was the sample amplified by forward primer of IgG4. All of isotypes used the same reverse primer. The expected sizes of the PCR products were 211, 207, 346, and 210 bp for IgG1, IgG2, IgG3, and IgG4, respectively.

Supplement table 1. Primers used for IgG isotype identification