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Research Article

Nicotiana benthamiana as a potential source for producing anti-dengue virus D54 neutralizing therapeutic antibody

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ABSTRACT

Dengue virus (DENV), transmitted by mosquitoes, is classified into four serotypes (DENV1-4) and typically causes mild, self-limiting symptoms upon initial infection. However, secondary infection can lead to severe symptoms due to antibody-dependent enhancement (ADE). To address this, anti-DENV antibodies are being developed with the goal of neutralizing infection without ADE activity. Previous attempts using a 54_hG1 antibody from CHO-K1 mammalian cells resulted in ADE induction, increasing viral infection. This study aimed to express the D54 monoclonal antibody in *Nicotiana benthamiana*. The plant-produced antibody had a similar neutralizing profile to the previous 54_hG1 antibody. Notably, the ADE activities of the plant-derived antibody were successfully eliminated, with no sign of viral induction. These findings suggest that *N. benthamiana* could be a source of therapeutic DENV antibodies. The method offers several advantages, including lower ADE, cost-effectiveness, simple facility requirements, scalability, and potential industrial-scale production in GMP facilities.

1. Introduction

Dengue fever is a mosquito-borne disease spread by *Aedes aegypti* and *Aedes albopictus*. Disease incidence has risen dramatically in recent decades, with reported cases increasing roughly tenfold from 505,430 in 2000 to 5.2 million in 2019 [1]. It can affect people of any age, including infants, adults, and the elderly [2], and has a concerning fatality rate of 20 % [3] if left untreated. According to the 2009 WHO classification, dengue fever is divided into two main types: non-severe dengue and severe dengue. Non-severe dengue can be further sub-categorized into dengue with a warning sign ($D + W$) and dengue without a warning sign (D-W) [4]. With urbanization and global warming promoting mosquito expansion, the global population at risk of dengue fever is projected to reach around 6 billion people by 2080 [5].

Dengue virus (DENV) is a single-stranded positive RNA virus from the Flaviviridae family. DENV comprises four serotypes (DENV1-4) [6], with closely related amino acid sequences. Previous research found that up to 30–35 % of the amino acid sequences varied between the four serotypes. There is evidence that primary DENV exposure causes variety of symptoms, the most common of which are non-severe chills, high fever, headache, and muscle pain [7]. In addition, DENV infection can be self-limited by producing antibodies against the viral spike protein and neutralizing the virus. However, secondary exposure to other serotypes can exacerbate the infection. Cross-reactivity of previously generated antibodies may enhance DENV entry into the cells via antibody-dependent enhancement (ADE), increasing viral replication and leading to severe disease forms, known as dengue hemorrhagic fever or dengue shock syndrome. These serious manifestations require an immediate diagnosis and treatment [8,9].

There is currently no specific treatment for DENV infection. Antipyretics, antiviral drugs and fluid replacement therapy are the most common symptomatic and supportive management options. Despite

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these measures, the fatality rate remains high, particularly with dengue hemorrhagic fever, reaching approximately 10–15 % without proper treatment [5]. Thus, there is an urgent need for effective antivirals to combat DENV infection. Laboratory-produced monoclonal antibodies (mAbs) against DENV have shown promise in limiting viral replication and infection [10–13]. However, antibody development is hindered by ADE and the lack of viral neutralization properties, which can lead to severe dengue [10–12]. To the best of our knowledge, only three anti-DENV mAbs are currently in clinical development. VIS513, a humanized IgG1 mAb targeting domain 3 of the dengue E protein [13], is being investigated for efficacy and safety in phase 2 clinical trial (NCT05418101). Meanwhile, the dengue mAbs AV-1 (NCT04273217) and Dengushield (NCT03883620) are undergoing safety assessments in phase 1 human trials [8].

Mammalian cell lines, mainly Chinese hamster ovary (CHO) cells, are widely utilized in commercial biopharmaceutical production [14]. Even so, their use is associated with high operating costs and sophisticated manufacturing facilities. Hence, there is a pressing demand to develop cost-effective methods for producing therapeutic antibodies. Plant-based expression systems are an appealing alternative for anti-DENV mAb production, as they provide lower manufacturing costs and easier scalability with simpler facilities. Additionally, these systems can carry out post-translational modifications crucial for mAb conformation and function [15]. While prior studies has explored into using plants to produce recombinant virus-like particles for dengue vaccine development [16–18], there is still a lack of data on plant-based therapeutic antibodies capable of neutralizing DENV infection. Therefore, further research in this area is required.

Sasaki et al. and colleagues prepared antibody-producing hybridomas from peripheral blood mononuclear cells of DENV-infected patients by fusing with human myeloma SPYMEG, resulting in hybridoma cells of anti-DENV mAb clone D23-1G7C2 (or 54_hG1) that can neutralize all four serotypes [11]. Subsequently, the gene encoding the D54 mAb was transferred into the CHO-K1 mammalian expression system. However, despite the therapeutic potential of mAb, its applications were limited by ADE activities, leading to increased infection rates [11, 12,19].

Given the existing knowledge gap and the high manufacturing costs associated with CHO-produced mAbs, there is an urgent need for alternative production platforms to develop effective neutralizing treatments. The current study aims to address this by producing anti-DENV D54 mAb using the *N. benthamiana* plant expression system. The heavy and light chain genes were introduced into the plant using *Agrobacterium tumefaciens* transient infection method. The expression level was optimized, and the plant-produced mAb was purified and characterized. The ADE and neutralizing properties were compared to those of the CHO-K1-produced mAb (54_hG1). The results of this study could establish plants as a promising platform for producing therapeutic mAbs against DENV, potentially leading to reduced production costs, enhanced scalability, and simplified manufacturing processes.

2. Materials and methods

2.1. Experimental research involving plants

The wild type *N. benthamiana* seeds used in this study were provided by Dr. Supaart Sirikantaramas from the Faculty of Science, Chulalongkorn University. Study protocols were reviewed and approved by the internal Chulalongkorn University-Institutional Biosafety Committee (CU-IBC) to adhere to Biosafety guidelines for modern biotechnology. All methods were conducted in line with the relevant guidelines, regulations, and legislation.

2.2. Gene construct

Sasaki et al. previously constructed 54_hG1 mAb using the CHO-K1 mammalian expression system [11]. The gene coding sequences of 54_hG1 heavy and light chain were retrieved (GeneBank accession numbers AB776836 and AB776837). In the current study, the variable regions of both the light (V_L) and heavy (V_H) chains were amplified by PCR using primers listed in Table 1. The V_H and V_L were fused with a murine leader sequence at the N-terminus at the *Xho*I site. The resulting V_H and V_L (with signal peptide) were digested with *Xba*I/*Bmt*I and *Xba*I/*Afl*II restriction enzymes, respectively, and then ligated with the constant regions of human IgG1 heavy chain (C_H1-C_H2-C_H3) or kappa chain (C_L). The D54 heavy and light chain genes were subsequently inserted into the pBY2eK expression vector [22]. Fig. 1 illustrates a schematic representation of the expression vector construct for the plant-produced D54 anti-DENV mAb.

Next, the heavy and light chain expression vectors were individually transformed into *A. tumefaciens* strain GV3101 using electroporation. For clone selection, a selective media supplemented with 50 µg/mL gentamycin, 50 µg/mL kanamycin, and 25 µg/mL rifampicin was used. After that, positive clones were verified by PCR and sequencing analysis.

2.3. Expression time cross experiment

Agrobacterium tumefaciens containing the heavy and light chains of D54 were infected into *N. benthamiana*. To optimize antibody expression, a time course experiment was performed. Bacteria were grown at 28 °C in antibiotic-selective media, and overnight cultures were diluted with infiltration buffer (10 mM 2-[N-morpholino] etanesulfonic acid (MES) and 10 mM $MgSO_4$, pH 5.5) until the absorbance (OD_{600}) reached 0.2. The heavy and light chains were co-delivered in equal ratios (1:1) into wild type *N. benthamiana* leaves via syringe infiltration. Infiltrated plants were maintained at 25 ± 2 °C with a 16 h photoperiod/ 8 h dark period cycle. Leaf samples were harvested at 2-, 4-, 6-, 8- and 10-days post-infiltration (dpi) to investigate leaf morphology, then ground in PBS and centrifuged to collect the supernatant.

For large-scale production, the vacuum infiltration technique was performed. The leaves were collected at optimal harvest time and extracted with PBS, as previously described above. The total protein concentration was determined using the Bradford protein assay, whereas antibody concentration was measured using the ELISA method. Proteins were separated by SDS-PAGE on a 4–15 % gradient gel, and the protein pattern was visualized with InstantBlue® Coomassie Protein Stain (Abcam, USA). The protein integrity and size were assessed by western blot analysis probed with anti-human gamma (1:10,000) and kappa (1:2500) detection antibodies.

2.4. Protein purification

Protein A resin (Amintra, Expedon, Cambridge, UK) was utilized to purify the anti-DENV mAb. The infiltrated leaves were extracted with PBS at a 1:2 (w/v) ratio and homogenized using a blender. The crude extract was centrifuged at 13,000 rpm for 10 min and clarified with 0.45 µm filter paper. The supernatant was loaded onto the protein A column and then washed with PBS. The anti-DENV antibody was eluted with an elution buffer (0.1 M citrate buffer at pH 2.7) and immediately mixed with a neutralizing buffer (1.5 M Tris–HCl pH 8.8) to adjust the pH to 7.4. The purified antibody was then dialyzed with PBS to remove excess salt and concentrated using Amicon ultra-centrifugal filter (Merck, Massachusetts, USA).

2.5. ELISA protocol for anti-DENV titer determination

The level of D54 expression was measured by ELISA, as described elsewhere [14]. In brief, a 96-well plate was coated with anti-human IgG-Fc fragment (Abcam, Cambridge, UK) (1:1000) in PBS and incubated for 2 h at 37 °C. After incubation, the plate was washed with PBS containing 0.05 % tween (PBS-T) and blocked with 5 % (w/v) skim milk in PBS for 1 h at 37 °C. Then, various concentrations of standard

Table 1
Primer sequences used for the construction of D54 mAb.

Primer name	Amino acid sequence (5′ to 3′ end)
XhoI_D54_VH_F	CCTCGAGCTGGTGCAGTCTGGGGCTG
BmtI_IgG1_D54_HC-R	GGTGCTAGCGGAGGAGACGGTGACGAGGGTAGAGACGGTGACCATTGTTCC
XhoI_D54_VL_F	CCTCGAGCCTGCCCCGTGTCTGGG
AflII_IgG1_D54_LC-R	CTTAAGCTGCTCGTCGGAGGGGGGGAAGATGAAGACGGAGGGGGCGGCGACGGTGACGGTCACCTTGGTCCC

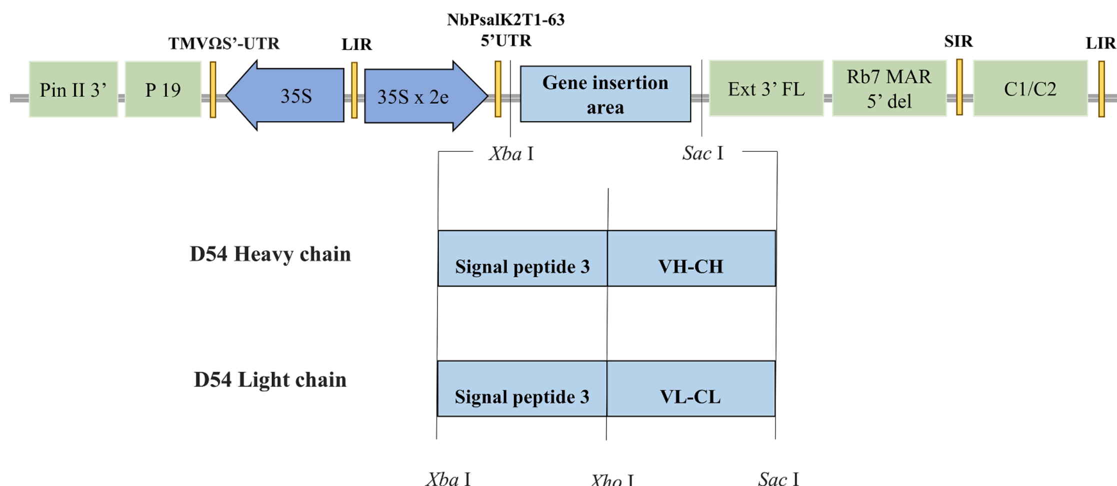

Fig. 1. The diagram of pBY2eK expression vector for D54 MAb. Pin II 3′: Potato proteinase inhibitor II terminator, P19: P19 silencing suppressor from Tomato Bushy Stunt Virus (TBSV), TMVΩ 5′-UTR: 5′ untranslated region (UTR) of Tobacco Mosaic Virus Ω, P35S: 35S promoter from Cauliflower Mosaic Virus (CaMV), P35Sx2e: 35s promoter from Cauliflower Mosaic Virus with duplicated enhancer, NbP 5′: 5′ UTR of *Nicotiana* photosystem I reaction center subunit psa K, XbaI and SacI: restriction enzyme sites for cloning gene of interest, VH-CH: heavy chain of anti-DENV gene, VL-CL: light.

anti-human IgG_1 kappa isotype were added to construct the calibration curve. A two-fold serial dilution of the protein sample was prepared and added to the reaction well. Horseradish peroxidase (HRP)-conjugated anti-kappa antibody (1:2500) in PBS was used as the detector reagent. The plate was washed with PBS-T, and the color was developed using TMB substrate. Finally, the reaction was stopped with 1 M H_2SO_4, and the absorbance was read at 450 nm.

2.6. N-glycan analysis

The analysis of N-glycans was performed as described in previous study [14]. The heavy chain of the purified anti-DENV antibody was separated from the full-length antibody by SDS-PAGE under reduced conditions, subsequently S-alkylated and digested with trypsin. Then, the glycoproteins and peptides were analyzed by LC-ESI-MS. Briefly, samples were captured on a pre-column (30 × 0.32 mm Aquansil C18, Thermo Scientific, Massachusetts, USA) and run through an analytical column (100 × 0.18 mm Biobasic C18, Thermo Scientific, Massachusetts, USA). The Q-TOF Ultima Global mass spectrometry was used to detect the m/z ratio of a positive ion in the range of 500–1600 (Water, Massachusetts, USA).

2.7. Focus reduction neutralization test (FRNT)

The cross-neutralization against DENV was examined using the FRNT assay, as described earlier [20]. Vero cells were seeded in 96-well culture plate at 2.5×10^4 cells/well. Two-fold serial dilutions of mAbs (starting from 64 μg/mL) were mixed with DENV and incubated for 1 h at 37 °C. The virus-antibody mixture was then transferred to Vero cell seeded plate and incubated for another 2 h. The infected cells were overlaid with a mixture of 2X MEM, 2 % carboxymethyl cellulose, and 3 % FBS and incubated for 2 days for DENV4 and 3 days for DENV1, 2, and 3. The cells were fixed with 3.7 % formaldehyde followed by permeabilization with 0.1 % Triton X-100. Next, the cells were incubated with anti-DENV mAbs for 1 h at 37 °C, and then with Alexa Fluor® 488-conjugated goat anti-human IgG ($H + L$) (1:1000) for 1 h at 37 °C. The foci numbers were counted using a fluorescence microscope. The neutralization potential was calculated as the percentage reduction in the number of foci for each mAb concentration when compared to a negative control. In this experiment, a virus-PBS mixture was used as a negative control.

2.8. Antibody dependent enhancement assay

In this study, we utilized an antibody dependent enhancement (ADE) assay to evaluate ability of plant-derived D54 mAb to neutralize infections caused by all four dengue virus serotypes (DENV 1-4) without promoting infection enhancement, which is an undesirable effect. The ADE activity of the 54_hG1 mAb, produced in mammalian systems, was also assessed. The FcγRII-containing human K562 erythroleukemia cells were employed to investigate ADE, as previously described [20]. Briefly, D54 and 54_hG1 were serially diluted four-fold in a poly-l-lysine coated plate, then mixed with DENV for 2 h at 37 °C before adding the K562 cells at 2.6×10^6 cell/mL. Plates were further incubated for 2 days. Following incubation, the cells were washed three times with PBS, air-dried, and fixed in acetone:methanol (50:50) for at least 30 min at -20 °C. After that, cells were incubated with anti-HuMAb overnight at 4 °C, followed by the addition of HRP-conjugated goat anti-Human IgG (1:250) as a secondary antibody and incubated for 1 h at 37 °C. DAB staining was used to develop the signal of infected cells.

To evaluate ADE activity, the number of infected cells at each mAb concentration was counted and compared to the number of infected cells in the negative control. Criteria for determining antibody neutralizing or enhancing activity were established based on the average plus standard deviation (SD) of the percentages of infected cells obtained from the six negative controls, which served as the baseline. An increase in the percentages of infected cells above the baseline indicates ADE, while a decrease below the baseline suggests neutralization activity [21].

3. Results

3.1. Gene construction and optimization of the time course for protein expression

To produce the D54 mAb, we synthesized the genes encoding the variable heavy chain and variable light chain using primers in Table 1 and incorporated into the constant regions of human IgG1. We also added a signal peptide (SP3) at the N-terminus to aid in protein localization and enhance production yield [22,23]. Following confirmation through sequencing analysis, we transformed the resulting vectors into the *A. tumefaciens* strain GV3101. Bacteria containing anti-DENV heavy and light chain expression vectors were then cultured overnight and co-infiltrated at a 1:1 ratio.

In this study, we optimized protein expression by varying the time-post-infiltration (2, 4, 6, 8, and 10 dpi). The amount of crude protein in the leaf extracts was quantified using the Bradford protein assay and the ELISA method. Protein expression was visually observed after 2 dpi, displayed as leaf necrosis (Supplementary Fig. S1). The highest level of anti-DENV mAb expression was detected on 2 dpi, with a maximum yield of 0.22 mg/g fresh weight (Fig. 2). Based on these results, 2 dpi was chosen as the optimum period for protein expression.

3.2. Protein purification

Following time-course optimization, protein production was scaled up. Six-week-old *N. benthamiana* plants were transfected using the vacuum infiltration method. Leaves were collected after 2 days and extracted with PBS. Plant crude extract was purified on a protein A column, and affinity-purified D54 mAb was eluted and concentrated using an Amicon ultra centrifugal filter. The yield of plant-purified anti-DENV was determined to be 0.34 mg/g fresh weight by the Bradford assay and 82.42 μg/g fresh weight by the ELISA method.

Next, SDS-PAGE and immunoblot analyses were used to assess the protein size and integrity (Fig. 3 and Supplementary Fig. S2). Approximately 5 μg of purified D54 mAb was loaded onto the gel, and the results are depicted in Fig. 3A, stained with InstantBlue® (Abcam, Cambridge, UK). Under non-reduced condition, a protein band was detected at around 150 kDa corresponding to the size of a fully assembled IgG. Meanwhile, under reduced condition, bands at 25 and 50 kDa were observed, corresponding to the sizes of light and heavy chains, respectively. This confirmed that the D54 mAb was correctly assembled into an IgG molecule. Additionally, the heavy and light chains of D54 mAb were identified by western blotting using the anti-human kappa and anti-gamma antisera, as shown in Fig. 3B and C. The variable heavy and light chain sequences of plant-produced D54 mAb and 54_hG1 were aligned using the Clustal Omega multiple sequence alignment tool, with results provided in the supplementary information (Supplementary Figs. S3, S4).

3.3. N-glycan analysis

The N-glycan profile was evaluated by LC-ESI-MS. After SDS-PAGE, the constant region of the heavy chain was extracted from the gel and subjected to trypsin digestion. Peptide sequence “EEQYNSTYR” was isolated for analysis. As shown in Fig. 4, the chromatogram revealed the abundance of high mannose (M) content, a characteristic of protein glycosylation in *N. benthamiana* at the endoplasmic reticulum. Likewise, a complex glycan combination containing N-acetylglucosamine (GlcNAc) with xylose (X) and fucose (F) residues were identified, indicating mature glycan processing typical of the medial and trans Golgi complex. The abundance of glycan structures could be ranked as follows: Man9, Man8, and GnGnXF. Trace amounts of other glycoforms, including MM, Man4, Man5, Man7, and GnMXF were also detected.

3.4. Cross-neutralizing activity of D54 and 54_hG1 against DENV

The cross-neutralizing activity of D54 mAb against the four DENV serotypes was further assessed and compared with that of the CHO-K1 cell-expressed 54_hG1 mAb. Overall, D54 mAb demonstrated notable cross-neutralizing activity against all serotypes, comparable to the neutralizing activity of 54_hG1 mAb. DENV2 and DENV4 were completely neutralized at 64 μg/mL of D54 mAb, whereas DENV1 and DENV3 were neutralized at approximately 60 % and 90 %, respectively (Fig. 5). Aside from DENV1, the FRNT50 of D54 against the other serotypes (DENV2, DENV3, DENV4) were almost identical to that of 54_hG1, as shown in Table 2. Thereby, the results suggest that the cross-neutralizing activity of D54 mAb against all four DENV serotypes is functionally robust and acceptable.

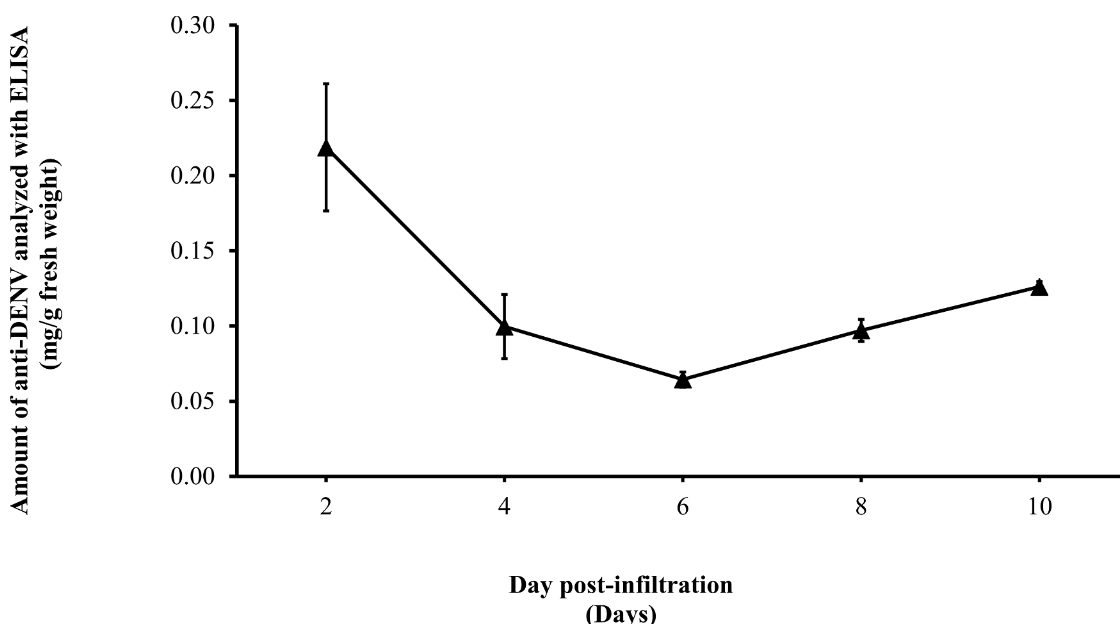

Fig. 2. The yield of plant-expressed D54 was determined by ELISA after 2, 4, 6, 8, 10 days post-infiltration (DPI).

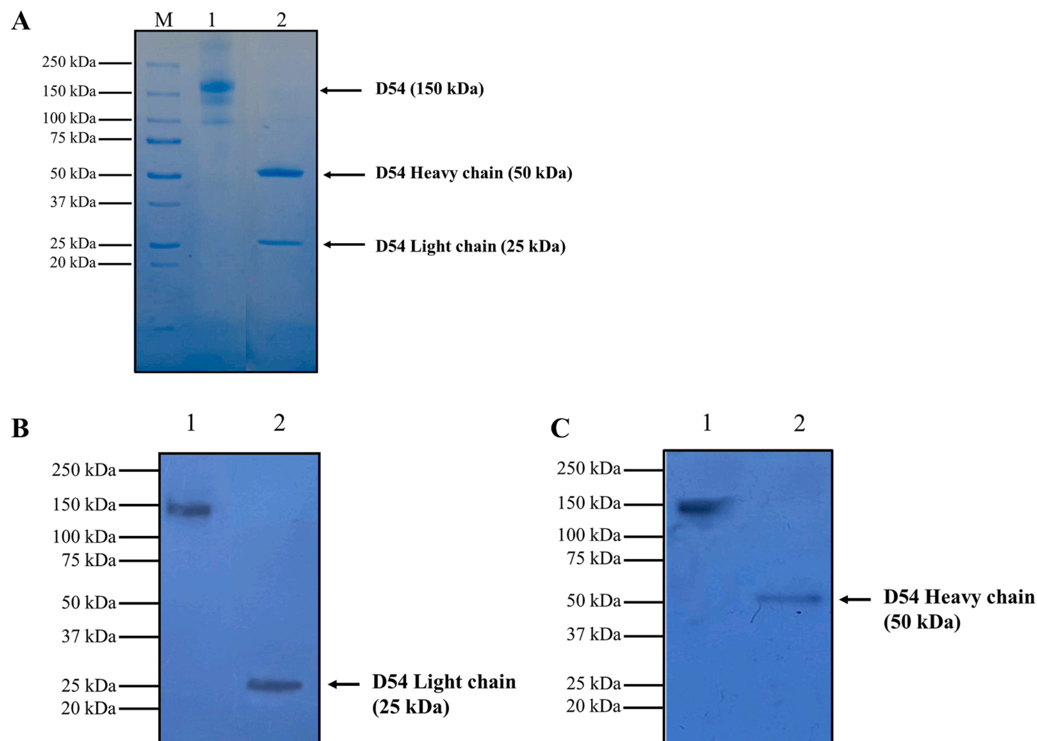

Fig. 3. SDS-PAGE and Coomassie staining of D54 after protein A affinity column purification (A). Western blot analysis with anti-human kappa for light chain detection (B) and anti-human gamma for heavy chain detection (C). M: protein marker, Lane 1: non-reduced D54 mAb, and Lane 2: reduced D54 mAb.

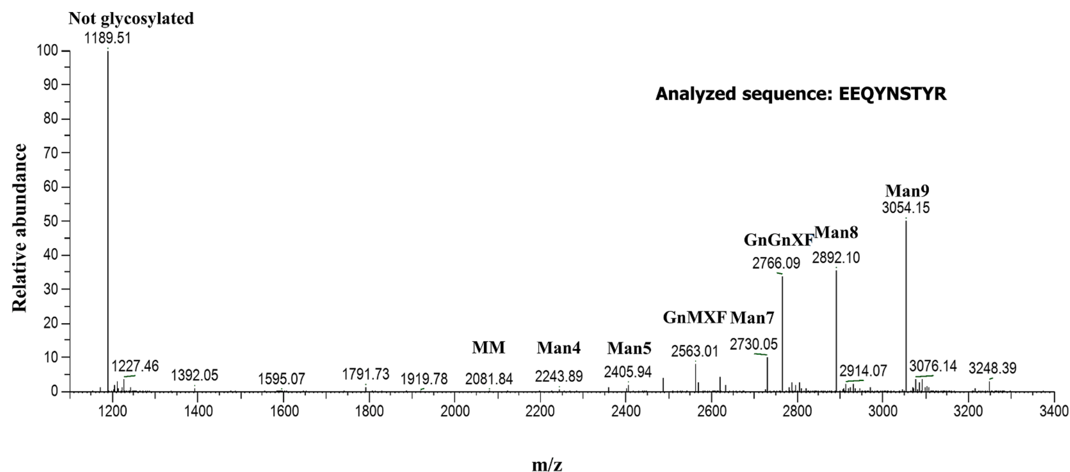

Fig. 4. N-glycosylation analysis of D54 heavy chain at the peptide sequence "EEQYNSTYR". M or Man: mannose, Gn: N-acetylglucosamine, X: xylose, and F: fucose.

3.5. ADE of D54 and 54_hG1 on DENV infected K562 cells

The ADE activity of both D54 expressed in *N. benthamiana* and 54_hG1 expressed in mammalian cells against DENV infection was tested on FcγRII-containing K562 cells. Infected cells were counted from three microscopic fields at 10X magnification, and the average count was multiplied by 153.86 to determine the total number of infected cells count per well. The cut-off value to differentiate between the neutralizing or enhancing activities was calculated from the average plus SD of the percentages of infected cells obtained with the six negative controls. Results indicated that D54 mAb effectively neutralized DENV1-DENV4 serotypes at higher mAb concentrations, with no significant increase in the number of infected cells observed across all four serotypes (Fig. 6). In contrast, except for DENV2, 54_hG1 demonstrated high enhancing activity in DENV-infected FcγRII-containing K562 cells. Specifically, DENV1 infection increased in the presence of 54_hG1 at concentrations of 100 μg/mL, 25 μg/mL, and 6.25 μg/mL, whereas DENV3 and DENV4 infection surpassed the enhancing baseline at concentrations of 25 μg/mL and 6.25 μg/mL, respectively. Taken together, plant-produced D54 mAb exhibited no ADE activity, thus reducing the risk of ADE.

4. Discussion

Currently, management of DENV infection is mainly based on symptomatic treatment and supportive care, which are insufficient in cases of severe dengue. Patients without access to adequate treatment have a mortality rate of 10–15 % [21]. Many researchers are currently working to develop mAbs against DENV for the treatment of severe dengue, such as dengue hemorrhagic fever or dengue shock syndrome. The proposed mechanism mimics host immune activity in order to

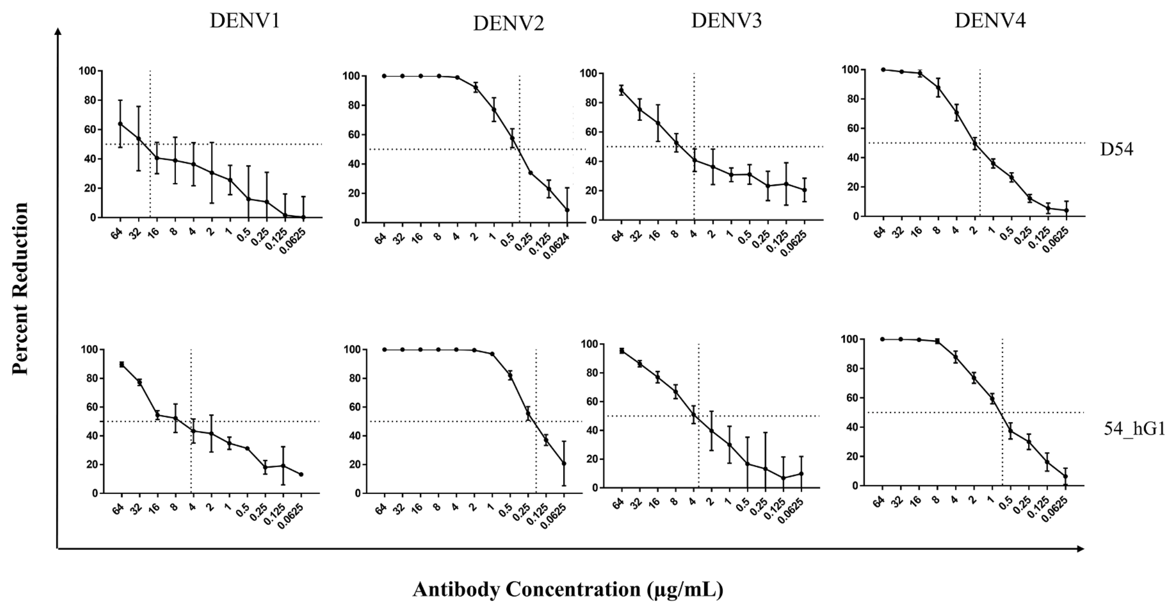

Fig. 5. Cross-neutralizing against DENV1-DENV4 of D54 and 54_hG1. Neutralizing activity against DENVs was determined from the representative of at least two independent experiments. Dotted line indicated 50 % neutralizing activity. Data are presented as mean ± SD.

Table 2
FRNT50 of D54 and 54_hG1 against DENV1-DENV4.

MAbs	FRTN50 in µg/mL (95 %CI)			
	DENV1	DENV2	DENV3	DENV4
D54	20.67 (11.13–49.15)	0.30 (0.34–0.43)	4.09 (2.79–6.04)	1.65 (1.48–1.83)
54_hG1	4.40 (3.20–6.11)	0.18 (0.17–0.2)	3.29 (2.40–4.12)	0.68 (0.61–0.76)

inhibit viral replication and infection [5,10]. Nonetheless, ADE remains a "point of concern" for neutralizing antibody development. Non-neutralizing antibodies and neutralizing antibodies at sub-neutralizing concentrations can both promote ADE [24]. These can be seen because secondary infection with heterogeneous serotypes can result in higher viral entry into the host cell. This phenomenon promotes viral replication, leading to poor therapeutic outcomes [25].

The majority of anti-DENV mAbs are typically manufactured using mammalian cell expression systems (i.e., CHO cells), a well-established platform for biopharmaceuticals production. However, this platform requires high maintenance costs and demands sophisticated operation techniques and manufacturing facilities [26]. Although other systems including *E. coli*, yeast, and insect cells have been used, they are unable to express heterogenous proteins with complex post-translational modifications [27]. Plant expression systems offer a promising solution for anti-DENV mAb production. Among these, *N. benthamiana* stands out as one of the most well-characterized plants for recombinant proteins

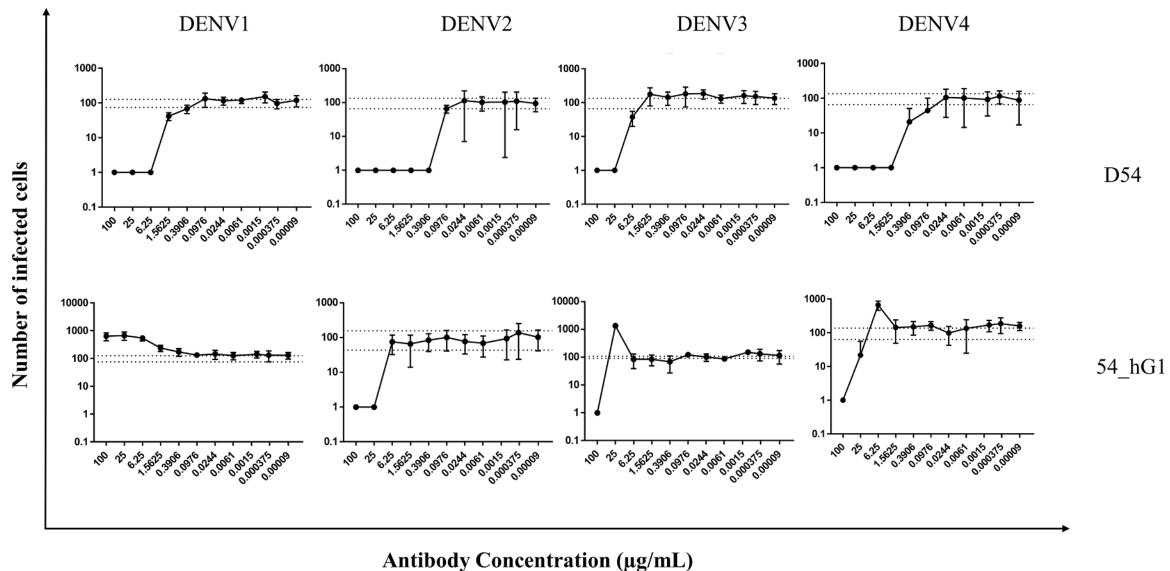

Fig. 6. Antibody-dependent enhancement (ADE) of D54 and 54_hG1 against DENV1-DENV4. Neutralization and enhancement activity against the four DENV serotypes in FcγRII-containing K562 cells. The number of infected cells (log10) from each antibody concentration was used to determine the enhancing activity. Dotted lines represent the mean of infected cells of no antibody control ± SD. The value above baseline was considered as ADE, and the value below baseline was considered as neutralization. Each data point represents the average of three replicates from a single experiment. The figure depicted the result from the representative of two experiments.

synthesis, like growth factors, enzymes, antibodies, and vaccines [28,29]. It provides unique benefits such as low operating costs, a rapid and simple production process, high reproducibility, ease of scalability, and the ability to express complex protein [30,31]. Given that dengue fever predominantly affects low-income tropical and subtropical countries [4], *N. benthamiana* emerges as an ideal candidate for large-scale production, aligning with good manufacturing practice (GMP) standards for pharmaceutical products [32].

Previous research established a stable clone of antibody 54_hG1 by transferring genes encoding D54 mAb into CHO-K1 cells. The D54 mAb or 54_hG1 effectively targets the fusion loop of domain II of the DENV envelope protein, exhibiting neutralizing activity against all four viral serotypes (DENV1-4). However, the mAb was challenged by the issue of ADE induction [19]. In response to cost constraints, efforts were made to develop the D54 mAb in plants. The protein yield obtained at 2 dpi amounted to 82.42 μg of D54 mAb per gram of leaf fresh weight (or 41.21 μg/mL of extraction buffer), as measured by ELISA. Western blot analysis detected bands at 150 kDa under non-reducing conditions and two bands at 25 kDa and 50 kDa under reducing conditions for the plant-produced D54 mAb. This data indicated the successful production of a complete anti-DENV antibody within the plant host. An FRNT assay conducted in Vero cells assessed the cross-reactivity and neutralizing activity of plant-produced D54 and CHO-K1-produced 54_hG1 mAbs. Both mAbs exhibited similar neutralization patterns across DENV serotypes, with potency was ranked as follows: DENV2, DENV4, DENV3, and DENV1. The findings are consistent with prior report regarding the stronger neutralizing activity of 54_hG1 (D23-1G7C2) against DENV2 [19]. Given the prevalence of DENV2 in severe dengue cases [33], the potent activity of D54 mAb against this serotype is advantageous. On the other hand, concerns about ADE virus induction must be considered, particularly with unbalanced neutralization. Hence, additional investigation on neutralization and enhancement activity in FcγRII-containing K562 cells was performed. Results confirmed the infection-enhancing activity or ADE potential of 54_hG1 mAb across all serotypes except DENV-2, making it unsuitable for therapeutic use. At sub-neutralizing concentrations, 54_hG1 mAb can induce ADE, resulting to enhanced viral entry and replication in host cells, ultimately poor clinical outcome [34]. Interestingly, the plant-produced D54 lacked enhancing activity against any virus serotype, suggesting a more favorable therapeutic outcome. These findings underscore the potential of plant-based mAbs like D54 to offer effective and safer treatments for DENV infections.

As mentioned above, ADE poses a significant hurdle in the development of therapeutic mAbs for DENV treatment. The mechanism involves the interaction between the antigen-antibody complexes (DENV-IgG from previous infection) and the Fc-gamma receptors (FcγR) on immune cells, leading to increased viral load via immune complex mechanism. ADE also enhances viral entry, fusion, and inhibits innate immunity [32,33]. Scientists are exploring various strategies to address this problem, including IgG subclass switching, gene mutation, and sugar modification. For example, Ramadhany et al. (2015) introduced mutations at position N297A of the anti-DENV mAb and switched the IgG subclass from IgG1 to IgG2 or IgG4. Their findings revealed varying outcomes: while IgG2 exacerbates ADE activity, the N297A mutation in IgG1 reduces ADE but may compromise protective activity [34]. In addition, introducing a LALA mutation into the Fc of anti-DENV mAb has been effective in completely abolishing ADE compared to the wild type mAb [5]. Other research also suggest that amino acid substitutions targeting FcγR binding can reduce NK-mediated antibody-dependent cellular cytotoxicity (ADCC), which is crucial for viral neutralization of mAbs [35–38].

Aside from Fc modifications, one potential strategy to eliminating ADE while preserving ADCC activity involves utilizing different expression systems. Prior studies compared mAbs derived from both mammalian cell lines and plants, revealing that plant-produced mAbs exhibited similar protective efficacy to their mammalian counterparts but differed in ADE activity [10,38]. Plant glycosylation was found to be more consistent and less heterogeneous compared to mammalian cells. One approached used a genetically modified ΔXF *N. benthamiana* to eliminate plant-specific glycosylation [39]. However, later publications reported that afucosylated mAbs had higher affinity for FcγR [40]. Therefore, we utilized wild type *N. benthamiana* in our study to express D54. The resulting plant-produced D54 mAb contained complex N-glycans with fucose and xylose residues, as predicted. Moreover, the abundance of GnGnXF glycoside may account for the lack of FcγRII binding [40], as this glycan structure has been associated with reduced receptor activity, thus mitigating ADE while retaining ADCC activity [36,37]. This theory is supported by the study of He et al., which demonstrated that plant-derived mAb against West Nile Virus with higher levels of GnGnXF displayed reduced binding to all FcγR subtypes and C1q, indicative of a lower ADE [41].

The *Agrobacterium*-mediated vacuum infiltration provides simplicity and speed in expressing target genes, introduced transiently into tobacco plants shortly before harvest. However, large-scale production faces a bottleneck requiring multiple transfections across generations. Both stable and transient methods encounter biomass growth barriers. Comparing these two methods, stable transformation demands strict plant cultivation due to transgenic contamination concerns, while transient agroinfiltration involves normal cultivation and plant bacterial transfection, thus lowering environmental control costs [42–44]. Previous study compared protein production costs in transient plant agroinfiltration and CHO cell expression [45]. Total capital investment for agroinfiltration of *N. benthamiana* plantlets to produce 300 kg/year of monoclonal antibodies is estimated at $122 million USD. In contrast, CHO cell production in a bioreactor may require double the investment for the same capacity. Plant gene transfer is commonly achieved via biolositic transformation or agroinfiltration [46]. Biolistic transformation, while versatile, lacks scalability due to the need for extensive tissue bombardment for large-scale protein production in plants. Moreover, substantial amounts of gold particles and helium gas are consumed, both being more expensive in the long-term compared to vacuum pump. On the contrary, vacuum agroinfiltration is faster, labor-efficient, and adaptable for large-scale operations, infiltrating hundreds of plants simultaneously. With agroinfiltration protein production can be achieved within weeks or months [42]. Protein yields from transient expression are generally higher than those from stable transformation [43]. Moreover, the transient expression with agroinfiltration is more appropriate for producing therapeutic anti-infective agents since it is more flexible for protein switching [44]. For scalability, this method is extensively used for industrial-scale production of therapeutic proteins, including the Baiya SARs-CoV-2 protein subunit vaccine, currently in phase 2 clinical trials [47]. The produced protein by this method is sufficiently supplied throughout clinical study. This method is also employed in the production of therapeutic proteins like anti-human PD-1 antibodies [48] and anti-botulinum neurotoxins [49] which are previously developed in our laboratory. Therefore, using agroinfiltration for the production of D54 mAb is more appropriate than stable transformation.

In terms of safety considerations regarding the use of plant-produced antibodies in humans, our group has conducted extensive studies on the safety of proteins produced in *N. benthamiana*. Previously, our investigations focused on the safety and toxicity of the subunit RBD vaccine, Baiya SARS-CoV-2 Vax 1 and Vax 2, in preclinical studies, revealing no significant toxicity [47,50]. Furthermore, our research team also compared the safety and efficacy of commercial anti-PDL-1 Atezolizumab with plant-produced Atezolizumab. The results showed significant inhibition of tumor growth without severe weight loss among mice treated with either plant-produced Atezolizumab, commercial Atezolizumab, or the control group (PBS) [51]. Other studies supporting the safety of antibodies produced in *N. benthamiana* have been reported. For instance, a plant-produced antibody vaccine for treatment of non--Hodkin's lymphoma demonstrated acceptable safety and immunogenicity results after completing a phase 1 clinical study [52]. ZMapp

antibody, another example derived from *N. benthamiana*, was developed by Kentucky BioProcessing for treatment of Ebola virus infection and received approval in 2014 [53]. More recently, ZB-06, an engineered human antibody (HC4-N) produced in tobacco plants, intended for the prevention of sexually transmitted diseases and contraception, exhibited good efficacy and safety in phase 1 clinical trial [54]. Looking forward, we aim to conduct a complete nonclinical study in the near future to further assess the safety profile of the D54 mAb.

5. Conclusion

In conclusion, this study presents a proof of concept for producing the D54 neutralizing mAb using a wild type *N. benthamiana* plant expression system. The plant-derived D54 mAb exhibits a DENV neutralizing profile comparable to the maternal clone 54_hG1 with a lower ADE effect. This finding is supported by glycan analysis, which revealed the presence of complex N-glycans containing fucose and xylose residues, as well as an abundance of the GnGnXF glycoside. This glycan profile leads to reduced FcγR binding, which is related to the ADE effect. Importantly, plant-based production offers several advantages, including lower production costs and scalability, and is suitable for deployment in low-income dengue prevalent regions. However, limitations of this study regarding the safety and efficacy profiles of the antibody in an *in vivo* animal model must be addressed in further study to fulfill preclinical data of therapeutic antibody.

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Supplementary material

Supplementary Material was uploaded separately on this submission

CRediT authorship contribution statement

Supaluk Krittanai: Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **Kaewta Rattanapisit:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Christine Joy I. Bulaon:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **Pannamthip Pitaksajjakul:** Writing – review & editing, Writing – original draft, Methodology. **Sujitra Keadsanti:** Writing – review & editing, Writing – original draft, Methodology. **Pongrama Ramasoota:** Writing – review & editing, Writing – original draft, Methodology, Conceptualization. **Richard Strasser:** Writing – review & editing, Writing – original draft, Methodology. **Waranyoo Phoolcharoen:** Writing – review & editing, Writing – original draft, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

W.P. is a founder/shareholder of Baiya Phytopharm Co., Ltd. K.R. and C.J.I.B are employees of Baiya Phytopharm Co., Ltd. The remaining authors declare no competing interests.

Data availability

No data was used for the research described in the article.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.btre.2024.e00844.

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