Plant-produced anti-dengue virus monoclonal antibodies exhibit reduced antibody-dependent enhancement of infection activity

Matthew Dent,Jonathan Hurtado, Amber M. Paul, Haiyan Sun, Huafang Lai, Ming Yang, Adrian Esqueda, Fengwei Bai, Herta Steinkellner and Qiang Chen

Correspondence Qiang Chen qiang.chen.4@asu.edu

1The Biodesign Institute and School of Life Sciences, Arizona State University, Tempe, AZ, USA
2Department of Biological Sciences, University of Southern Mississippi, Hattiesburg, MS, USA
3Department of Applied Genetics and Cell Biology, University of Natural Resources and Applied Life Sciences, Vienna, Austria

The mAb E60 has the potential to be a desirable therapeutic molecule since it efficiently neutralizes all four serotypes of dengue virus (DENV). However, mammalian-cell-produced E60 exhibits antibody-dependent enhancement of infection (ADE) activity, rendering it inefficacious in vivo, and treated animals more susceptible to developing more severe diseases during secondary infection. In this study, we evaluated a plant-based expression system for the production of therapeutically suitable E60. The mAb was transiently expressed in Nicotiana benthamiana WT and a DXFT line, a glycosylation mutant lacking plant-specific N-glycan residues. The mAb was efficiently expressed and assembled in leaves and exhibited highly homogenous N-glycosylation profiles, i.e. GnGnXF3 or GnGn structures, depending on the expression host. Both E60 glycovariants demonstrated equivalent antigen-binding specificity and in vitro neutralization potency against DENV serotypes 2 and 4 compared with their mammalian-cell-produced counterpart. By contrast, plant-produced E60 exhibited reduced ADE activity in Fc gamma receptor expressing human cells. Our results suggest the ability of plant-produced antibodies to minimize ADE, which may lead to the development of safe and highly efficacious antibody-based therapeutics against DENV and other ADE-prone viral diseases. Our study provides so far unknown insight into the relationship between mAb N-glycosylation and ADE, which contributes to our understanding of how sugar moieties of antibodies modulate Fc-mediated functions and viral pathogenesis.

INTRODUCTION

Dengue fever (DF) is a widespread disease caused by dengue virus (DENV) and is endemic to the tropical areas of Africa, Southeast Asia and South America. It is a mosquito-borne illness spread primarily by the urban-adapted species Aedes aegypti (Murray et al., 2013). DENV is a member of the family Flaviviridae, which includes Zika (ZIKV), West Nile (WNV), Japanese encephalitis and yellow fever viruses. Genetically, DENV consists of a single-stranded positive-sense RNA genome coding for a single ORF encoding a single polyprotein, which is proteolytically cleaved into three structural proteins [capsid, envelope (E) and premembrane/membrane(prM/M)] and seven non-structural proteins (NS1, NS2A/B, NS3, NS4A/B and NS5) (Burke & Monath, 2001). As in other flaviviruses, the E and prM/M proteins contain the majority of epitopes for antibody response in humans (Beltramello et al., 2010; de Alwis et al., 2011); and the E protein has the typical three-domain (EDI–EDIII) architecture: a central β-barrel EDI, an elongated EDII containing the fusion loop conserved in all flaviviruses, and a C-terminal immunoglobulin-like EDIII (Kuhn et al., 2002; Mukhopadhyay et al., 2005).

Together, the four DENV serotypes (DENV1–4) represent one of the largest global disease burdens to date, with over 3 billion people at risk for infection and ~390 million infections in tropical and subtropical regions of the world annually (Murray et al., 2013). DENV can cause a spectrum of clinical manifestations, from mild fevers to fatal vascular leakage. Primary infection with one serotype usually produces asymptomatic, self-limiting DF. However, secondary infection with another DENV serotype increases the risk of developing severe disease, including life-threatening
vascular leakage syndrome, known as dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS) (Halstead, 2007; Rothman, 2004). The frequency and severity of DENV epidemics have increased significantly in recent years, with a higher incidence of DHF/DSS in regions that used to have outbreaks of mild disease (Murphy & Whitehead, 2011; Rico-Hesse et al., 1997). This may be due to certain factors such as global urbanization, trade and international travel, which can promote the geographic expansion of the DENV mosquito vectors and co-circulation of the four DENV serotypes in the same region (Kyle & Harris, 2008; Wilder-Smith & Gubler, 2008). Despite global expansion of DENV epidemics, no licensed human therapy is currently available to treat DENV-induced disease in humans, albeit a chimeric tetravalent live attenuated vaccine (Dengvaxia) has recently been licensed in five countries (Pang, 2016).

mAbs have been at the forefront of treatments for a wide array of diseases due to their specificity, potent efficacy and relative ease of production in a number of platforms (Chen & Davis, 2016). The tobacco plant, N. benthamiana, has been used to produce recombinant proteins ranging from enzymes to vaccines and mAbs (Arntzen, 2015; Bendandi et al., 2014). This may be due to certain factors such as ADE activities. IgG antibodies carry a single, conserved N-glycosylation site at the Fc domain, which is decorated with a core structure consisting of three mannose and two N-acetylgalactosaminyl residues, i.e. GnGn structures, irrespective of whether they are expressed in plants or mammalian cells (Chen, 2016). However, further diversification of glycans differs. IgGs produced in mammalian cells can be decorated with β1,4-galactose (A) and α1,6-fucose (F₆) and exhibit a mixed population of glycan species. By contrast, plant-expressed IgGs carry β1,2-xylene (X) and α1,3-fucose (F₃) in a single predominant glycoform, i.e. GnGnXF₃ (Chen, 2016). Concerns were raised that mAbs produced in plants might elicit unwanted immune responses to plant-specific glycans. To overcome this potential challenge, ‘humanization’ of the N-glycosylation pathway in N. benthamiana has been carried out by knocking out/down genes for plant-specific glycan synthesis and introducing mammalian genes of glycoenzymes (Chen, 2016; Strasser et al., 2014). In this course, a N. benthamiana mutant (AXFT) was generated by RNA interference (RNAi) technology to silence the expression of the endogenous β1,2-xylotransferase and α1,3-fucosyltransferase genes (Strasser et al., 2008). Importantly, mAbs produced in this plant line have a homogenous GnGn glycan species, lacking plant-specific β1,2-xylene and core α1,3-fucose residues. As a result of glycoengineering, a portfolio of N. benthamiana lines has been generated with demonstrated capacity to produce mAbs with various distinct mammalian glycoforms and a high degree of homogeneity that cannot be matched by mAbs produced in mammalian cell cultures (Loos et al., 2014; Strasser et al., 2008). Since the binding of human IgG antibodies to FcyR is highly sensitive to the composition of N-linked glycans, the availability of such mutants is valuable for the evaluation of the impact of glycosylation on antibody properties, such as ADE activities.

Here, we investigated the impact of N-glycosylation on ADE of anti-DENV mAb (E60) (Pierson et al., 2007), a chimeric mAb against the DENV EDII fusion loop, by using a plant transient expression system. We were able to rapidly produce E60 at high levels in both WT and AXFT N. benthamiana plants. Plant-derived E60 (pE60) carried the expected N-glycoform with high uniformity (i.e. GnGnXF₃ and GnGn structures, respectively) and retained the potent neutralization activity of E60 produced in mammalian cell cultures (mE60) against both DENV-2 and DENV-4. In contrast to an aglycosylated E60 (aE60) variant, pE60 variants carry N-glycans that bind C1q and a subset of FcγRs, potentially preserving CDC and ADCC activity. Interestingly, pE60 exhibited reduced ADE activity compared with mE60 in an in vitro cell-based assay. This study demonstrates that pE60 may be a preferred therapeutic candidate against DENV compared with mE60 or aE60, as pE60 induces ADE and has no in vivo therapeutic activity, and aE60 loses both ADC and CDC activity and may have a shorter half-life in circulation. This study may lead to the development of safer and more efficacious antibody-based therapeutics against DENV and other viral diseases that are
prone to ADE, and facilitates our understanding of how N-glycosylation affects ADE and DENV pathogenesis.

RESULTS

E60 mAb expression and assembly in N. benthamiana

The coding DNA sequence of the heavy chain (HC) variable region (\(V_H\)) and light chain (LC) variable region (\(V_L\)) of E60 was fused to the corresponding coding sequence of the human IgG1 HC constant region (\(C_H\)) and kappa LC constant region (\(C_L\)), respectively. The resulting coding sequences of E60 HC and LC were cloned into MagnICON-based plant expression vectors (Giritch et al., 2006) and transformed into Agrobacterium tumefaciens. A. tumefaciens strains that contained E60 HC and LC were agroinfiltrated into WT and \(\Delta XFT\) N. benthamiana leaves (Chen et al., 2013; Leuzinger et al., 2013). The genes of E60 HC and LC were also cloned into vector pcDNA3.1 and transfected into Chinese hamster ovary (CHO) cells for the production of mE60. Western blot analysis was performed to evaluate the expression of the antibody. Fig. 1 shows the expression profile of pE60 produced in \(\Delta XFT\) (\(\Delta XFpE60\)) under reducing or non-reducing conditions. HC and LC exhibited the expected molecular size (Fig. 1a, b, lane 3) and fully assembled into the heterotetrameric equivalent to a positive control mAb (Fig. 1a, Lane 6). Similar results were also obtained for pE60 extracted from WT (WTpE60) N. benthamiana leaves (data not shown). ELISA showed a maximum expression of the antibody 8 days post agroinfiltration (DPI), with an average accumulation of 120 \(\mu\)g [g leaf fresh weight (LFW)]\(^{-1}\) (Fig. 2). This level is similar to that previously reported for non-codon-optimized mAbs produced in plants (Bendandi et al., 2010; De Muynck et al., 2010; Lai et al., 2010). Stable CHO cell lines that expressed mE60 with the expected size and assembly were established and confirmed by Western blot and ELISA analysis (data not shown).

Purification of pE60 from N. benthamiana leaves

pE60 was extracted and purified from leaves of WT and \(\Delta XFT\) plants with a two-step purification process consisting of ammonium sulfate precipitation and protein A affinity chromatography, a process that we previously developed for processing plant-produced anti-WNV mAbs (Lai et al., 2010, 2012). We were able to efficiently purify pE60 from \(\Delta XFT\) leaves to >90% purity (Fig. 3). Similar results were obtained with pE60 from WT plants and mE60 from tissue culture media of transfected CHO cells (data not shown). Purified \(\Delta XFpE60\), WTpE60 and mE60 were used for further functional studies.

N-Linked glycosylation pattern of pE60

It has been shown that the nature of N-linked glycosylation in the \(C_H\) domain of an antibody affects its pharmacokinetics, shelf stability and Fc-domain-mediated functions (Houde et al., 2010). Thus, we next examined the N-glycosylation of pE60 by liquid chromatography–electrospray ionization–mass spectrometry (LC–ESI–MS). WTpE60 exhibited the expected complex-type N-glycans that terminate with N-acetylglucosamine residues and carry plant-specific xylose and core fucose (GnGnXF; Table 1), typical for plant-produced IgGs (Loos & Steinkellner, 2012). \(\Delta XFpE60\) was shown to carry mammalian type GnGn structures lacking xylose and fucose (Table 1). Notably, both \(\Delta XFpE60\) and WTpE60 have a single predominant N-glycoform with high degrees of homogeneity.

Fig. 1. Western blot analysis of plant-produced E60. pE60 was extracted from \(\Delta XFT\) N. benthamiana leaves, separated on SDS-PAGE gels under reducing (a and b, lanes 1–3) or non-reducing (a, lanes 4–6) conditions, and blotted onto PVDF membranes. The membranes were incubated with a goat anti-human gamma chain antibody or a goat anti-human kappa chain antibody to detect light chain (a) or heavy chain (b). Lanes 1 and 4, plant-produced anti-WNV E16 mAb as a reference standard; lanes 2 and 5, protein sample extracted from un-infiltrated leaves; lanes 3 and 6, sample from leaves infiltrated with E60 constructs. HC, heavy chain; LC, light chain; (HL)\(_2\), assembled mAb with two light and heavy chains.

Fig. 2. Temporal expression pattern of E60 in N. benthamiana leaves. Total soluble proteins from WT plant leaves infiltrated with E60 constructs were extracted at 5–9 DPI and analysed by an ELISA that detects the assembled form of p60 mAb variants. Mean±SD of samples from three independent infiltrations are presented.
mE60 bound in a similar manner to DENV-2 E with a $K_D$ of 0.93. This result demonstrated that WTpE60 and mE60 were retained antigen-binding specificity, kinetics and affinity that are comparable to mE60.

Fig. 3. Purification of pE60 from N. benthamiana leaves. Total soluble proteins were extracted from ΔXFT plants on day 8 after agroinfiltration. p60 was purified and analysed on a 4–20% gradient SDS-PAGE gel under reducing (lanes 1–4) or non-reducing (lanes 6 and 7) conditions and visualized with Coomassie stain. Lane 1, total soluble protein extracted from leaves; lanes 2 and 6, pE60 purified from leaves; lane 3, blank lane; lanes 4 and 7, plant-produced anti-WNV E16 mAb as a reference standard. HC, heavy chain; LC, light chain; (HL)$_2$, assembled mAb with two light and heavy chains. One representative of several independent experiments is shown.

Antigen binding of pE60

The specificity of WTpE60 and ΔXFP60 in binding DENV EDII antigen was assessed by flow cytometric analysis of yeast cells that display DENV-2 EDI-EDII on their surface. Results showed that binding of pE60s to negative control yeast and binding of a negative control mAb (pGP1) to yeast displaying EDI-EDII only produced a basal level of fluorescence (Fig. 4). In contrast, incubation of WTpE60 or ΔXFP60 with yeast cells displaying EDI-EDII resulted in a new fluorescent peak, with the percentage of positive yeast and the mean fluorescence intensity similar to those of mE60 (Fig. 4). The binding kinetics and affinity of WTpE60 and ΔXFP60 to their antigen were also examined by ELISA in which DENV-2 E was immobilized. As shown in Fig. 5, WTpE60, ΔXFP60 and mE60 bound in a similar manner to DENV-2 E with a $K_D$ value of 13.64, 12.72 and 12.37 ng ml$^{-1}$, respectively ($P$ values of WTpE60 and ΔXFP60 as compared with mE60=0.96 and 0.93). This result demonstrated that WTpE60 and ΔXFP60 retained antigen-binding specificity, kinetics and affinity that are comparable to mE60.

Neutralization activity of pE60 against DENV-2 and DENV-4

The neutralization potential of WTpE60 and ΔXFP60 against DENV was examined by focus reduction neutralization test (FRNT) with immunostaining analysis (Paul et al., 2014). Results showed that although not significant, the mean $EC_{50}$ values demonstrated that ΔXFP60 was more potent in neutralizing DENV-2 than mE60, while WTpE60 was less potent in neutralizing DENV-2 (Table 2). The $EC_{50}$ value of ΔXFP60 neutralization against DENV-2 was similar to that of mE60, while WTpE60 neutralized DENV-4 infection slightly more potently compared with mE60 (Table 2). Overall, WTpE60 and ΔXFP60 retained potent neutralizing activity against infectious DENV-2 and DENV-4.

Table 1. N-linked glycans of E60 and E16 variants

<table>
<thead>
<tr>
<th>Major N-glycan species</th>
<th>WTpE60 (%)</th>
<th>ΔXFP60 (%)</th>
<th>mE60 (%)</th>
<th>ΔXFP16 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GnGnXF$_3$</td>
<td>70</td>
<td>10</td>
<td>95</td>
<td>95</td>
</tr>
<tr>
<td>GnX</td>
<td>10</td>
<td>95</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>GnGn</td>
<td>10</td>
<td>95</td>
<td>95</td>
<td>95</td>
</tr>
<tr>
<td>AGnF$_5$</td>
<td>50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AAF$_5$</td>
<td>40</td>
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</tbody>
</table>

Throughout this study, the presence of the different glycoforms was expressed in glycans as percentages. Note, ΔXFT is a N. benthamiana N-glycosylation mutant that decorates proteins with mammalian-type GnGn glycans. Glycans were annotated according to the ProGlyCan nomenclature (www.proglycan.com).

Antibody-dependent enhancement activity of pE60

Since N-linked glycosylation in the Fc region of an antibody affects its binding to FcγRs, it may consequently have an impact on ADE, a phenomenon that is relevant to disease pathogenesis of DENV (Halstead et al., 2010; Mehlhop et al., 2007; Morens, 1994). As such, we investigated whether plant-produced E60 would have an altered ADE profile in vitro compared with that of mE60. mE60 efficiently promoted ADE in K562 cells that express the human FcγRIIa (Fig. 6), a phenomenon observed in previous studies (Pierson et al., 2007). In comparison, both WTpE60 and ΔXFP60 displayed no ADE activity in human K562 cells, similar to that of the negative control mAb (Fig. 6). The differences in ADE activities of the three E60 variants are attributed to the differences in the glycosylation pattern as all three versions contain the identical protein backbone.

DISCUSSION

mAbs produced in mammalian cell culture have achieved remarkable pharmaceutical and financial success with several blockbuster therapeutic products. However, mammalian-cell-produced mAbs are prohibitively expensive, presenting challenges for their accessibility in many parts of the world (Shevitz et al., 2011). The high cost of mAbs currently on the market encourages the development of alternative production systems including plants. Indeed, plants have been shown to...
be a promising system to produce mAbs with comparable yield and quality, but with lower cost and shorter process development time (Bendandi et al., 2010; Chen, 2011; Chen & Lai, 2015; Chen et al., 2011; Giritch et al., 2006; He et al., 2012; Huang et al., 2010; Zeitlin et al., 2011). In addition to the traditional benefit of low cost, plant-host engineering has offered the capability of producing mAbs with targeted N-glycans, and therefore serves as a system to develop mAbs with superior efficacy and safety.

In this study, we investigated the feasibility of using plant-produced mAb glycovariants to develop therapeutic candidates against DENV while addressing ADE, which is a major impediment to the development of mAb-based therapeutics against DENV. Antibodies are a significant component of the host’s protective immune response against flavivirus infections. Previous studies have shown that a large proportion of human anti-DENV antibodies target EDII with variable neutralizing capability and are cross-reactive with other DENV serotypes (Dejnirattisai et al., 2015). However, several mAbs that target the fusion loop of EDII have been found to be both neutralizing and cross-reactive (Deng et al., 2011). Furthermore, neutralizing antibodies have been observed to recognize quaternary epitopes at the hinge

**Table 2. Neutralization of DENV by pE60 variants**

<table>
<thead>
<tr>
<th>mAb</th>
<th>DENV-2 EC(_{50}) (ng ml(^{-1})) ± SEM</th>
<th>P value</th>
<th>DENV-4 EC(_{50}) (ng ml(^{-1})) ± SEM</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>mE60</td>
<td>362.10±179.60</td>
<td></td>
<td>675.87±200.34</td>
<td></td>
</tr>
<tr>
<td>WTpE60</td>
<td>433.51±82.29</td>
<td>0.75</td>
<td>377.82±95.92</td>
<td>0.31</td>
</tr>
<tr>
<td>ΔXFpE60</td>
<td>230.77±71.60</td>
<td>0.57</td>
<td>658.57±14.03</td>
<td>0.94</td>
</tr>
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</table>

**Fig. 4.** Antigen binding of pE60 variants to DENV EDII. Recombinant yeast cells that display domain I-II of DENV-2 E protein and negative control yeasts transformed with empty vector were stained with WTpE60, ΔXFpE60, mE60 (positive control) or a plant-produced humanized mAb against Ebola virus GP1 protein (pGP1, negative control). Yeast cells were then processed by flow cytometry. Representative data from at least three independent experiments are shown. M1, gate set for background fluorescence from negative control yeasts stained with WTpE60; M2, fluorescence with intensities beyond that of M1.

**Fig. 5.** ELISA of DENV E protein binding by pE60 variants. Serial dilutions of pE60 variants were incubated with DENV-2 E protein coated on microtitre plates and detected with an HRP-conjugated anti-human gamma-HC antibody. Dilutions of mE60 and a generic human IgG were used as reference standards and negative controls, respectively. The OD\(_{450}\) (mean±SEM) values from three independent experiments are presented.
region between EDI and EDII on adjacent E proteins (de Alwis et al., 2012). E60 is a mAb that was developed with the E protein of WNV and was later found to be cross-reactive with the highly conserved fusion-loop in EDII, and efficiently neutralizes all four DENV serotypes (Balsitis et al., 2010; Oliphant et al., 2006). Therefore, E60 has the potential to be a desirable therapeutic mAb that is protective against all serotypes of DENV. However, mE60 produced by mammalian cells exhibits ADE during DENV infection both in vitro and in vivo (Balsitis et al., 2010). Due to its ADE activity, mE60 has no therapeutic activity and may even render treated subjects more susceptible to developing life-threatening DHF/DSS in secondary infection (Balsitis et al., 2010). In order for E60 to become an efficacious and safe human therapeutic agent against DENV, it must forego its ADE activity but retain its neutralization potency, effector function and stability in circulation.

Studies have shown that the N-linked glycosylation of a mAb affects its stability, FcγR and C1q binding, pharmacokinetics, effector function and efficacy (Houde et al., 2010). However, the underlying conserved fusion-loop in EDII, and efficiently neutralizes all four DENV serotypes (Balsitis et al., 2010; Oliphant et al., 2006). Therefore, E60 has the potential to be a desirable therapeutic mAb that is protective against all serotypes of DENV. However, mE60 produced by mammalian cells exhibits ADE during DENV infection both in vitro and in vivo (Balsitis et al., 2010). Due to its ADE activity, mE60 has no therapeutic activity and may even render treated subjects more susceptible to developing life-threatening DHF/DSS in secondary infection (Balsitis et al., 2010). In order for E60 to become an efficacious and safe human therapeutic agent against DENV, it must forego its ADE activity but retain its neutralization potency, effector function and stability in circulation. 

Fig. 6. Antibody-dependent enhancement of pE60 variants. Serial dilutions of E60 variants or a generic human IgG (negative control) were mixed with DENV-2 and added to FcγR-expressing K562 cells. Forty-eight hours later, cells were fixed, permeabilized and stained with anti-DENV E antibody 4G2 and analysed by flow cytometry for DENV infection of cells.

Plant-produced anti-dengue virus mAbs with reduced ADE activity lines that produce mAbs with various defined and uniform mammalian N-linked glycans, thus providing a unique system for evaluating the contribution of mAb carbohydrate moieties to Fc-mediated functions (Chen, 2016; Loos & Steinkellner, 2014). The availability of these plant lines also eliminates the concern of the potential immunogenicity and associated adverse effects of plant-specific glycans and provides the opportunity to produce mAbs with tailor-made N-glycosylation patterns to enhance the efficacy or safety through modulating their effector function. For example, anti-Ebola mAb 13F6 produced in ΔXFT N. benthamiana had no plant-specific N-glycans but contained only a single mammalian glycoform, i.e. GnGn (Strasser et al., 2008; Zeitlin et al., 2011). In contrast, mammalian-cell-produced 13F6 had a mixture of 3–5 glycoforms (Zeitlin et al., 2011). The lack of fucose and the high homogeneity of plant-derived mAbs have led to their higher affinity to FcγRIII, improved ADCC and enhanced potency against Ebola virus over the mammalian-cell-produced 13F6 (Zeitlin et al., 2011). The superior potency of plant-produced mAbs was further demonstrated in a challenge study with non-human primates, in which plant-produced mAbs were far more protective against a lethal Ebola challenge than those produced in mammalian cells (Olinger et al., 2012). In a remarkable and exciting development, ZMapp, a cocktail of three AXFT-produced anti-Ebola mAbs, was recently used to treat human Ebola patients and showed promising results (Check Hayden & Reardon, 2014; Chen & Davis, 2016). Similarly, we found that AXFT-derived E16 mAbs displayed enhanced WNV neutralization in comparison with their mammalian counterparts (Lai et al., 2014).

Notably, the interaction between Fc and FcγRs can have pathogenic consequences such as ADE, which has been implicated for the development of DHF/DSS. ADE occurs because sub-neutralizing antibodies or sub-neutralizing concentrations of antibodies form complexes with the infecting flavivirus that bind to FcγR-bearing cells, resulting in increased virus uptake and infection (Morens, 1994). For example, the ADE activity renders mE60 inefficient in vivo and unsafe despite its potent neutralizing activity against all DENV serotypes (Balsitis et al., 2010; Oliphant et al., 2006). Here, we show that WTpE60 and ΔXFP60 exhibited a single predominant expected N-glycoform with a high degree of homogeneity. Further characterization of these glycovariants indicated that they both retained specific binding to EDII antigen with similar kinetics and affinity and the potent neutralizing activity of mE60 against multiple DENV serotypes. Importantly, our results demonstrated that both WTpE60 and ΔXFP60 have reduced ADE activity on FcγR-expressing K562 cells, in contrast to mE60 that showed strong ADE activity. This indicates that unlike the AAΦΦ and AGΦΦΦ glycans of mE60, the plant complex-type N-glycans (GnGnXFΦ) or the glycoengineered form GnGn of pE60 do not induce ADE under our conditions. This suggests that the α1,6 fucose, the terminal β1,4-galactose (AA or AGn) or the combination of both in mE60 may contribute to the induction of ADE.
The relationship of mAb binding to various FcγRs and ADE is a complex one. While antibody binding to FcγRs seems to be a necessary step, the binding alone may not be sufficient to cause ADE as results have shown that the downstream signalling after FcγRs binding is critical for ADE induction (Boonnak et al., 2013; Huang et al., 2016; Rodrigo et al., 2006). Therefore, the impact of N-glycans of mAbs on ADE may not depend solely on their ability to enhance or eliminate general FcγR binding, but instead, may depend on their ability to promote or inhibit the preferential binding to a specific subset of FcγRs with signalling pathways that promote viral entry and/or replication. For example, preferential binding of DENV-immune complex to FcγRIIa or FcγRIIB leads to opposite effects: FcγRIIa led to the development of ADE, but FcγRIIB not only did not promote ADE but also constrained the ADE effect of FcγRIIa (Boonnak et al., 2013; Chan et al., 2011). While human FcγRI and FcγRIIa have both been implicated in the development of ADE, FcγRIIa has been found to be the dominant FcγR in ADE-mediated DENV infectivity compared with FcγRI in mast cells and cell-line-based experiments (Brown et al., 2006; Chawla et al., 2013; Mohamad Zamberi et al., 2015; Rodrigo et al., 2006, 2009). The role of FcγRIII in mediating ADE for DENV is uncertain, although it seems not to play a role in the ADE of primary human monocytes (Boonnak et al., 2011; Tirado & Yoon, 2003). Our previous studies with E16 (Hu-E16scFv-Cγ1) produced in N. benthamiana plants with predominantly high-mannose N-glycans had slightly enhanced binding to the low-affinity isoform (FcγRIII-F<sup>3286</sup>) of FcγRII and FcγRI, while maintaining similar binding to the high-affinity FcγRIII isofrom (FcγRIII-V<sup>158</sup>), compared with mammalian-cell-produced E16 (He et al., 2014). Plant-derived E16 with typical plant GnGnxF<sub>3</sub> glycans, however, showed reduced binding to all FcγRs (He et al., 2014). In comparison, ΔXF-produced E16 had enhanced binding to both isoforms of FcγRII, reduced binding to FcγRII and similar binding to C1q, compared with mammalian-cell-produced E16 (Q. Chen, unpublished data), which is in agreement with what has been observed for ΔXF-produced anti-Ebola mAb 13F6 (h-13F6ΔXF) by other investigators (Zeitlin et al., 2011). Our previous studies also demonstrated that while mammalian-cell-produced E16 enhanced the infection of K562 cells by WNV, all three N. benthamiana-produced E16 variants lost their ability to induce ADE (He et al., 2014). Even though ADE is not a critical issue for WNV pathogenesis, these results suggest a putative impact of N-glycan moieties on the ADE activity of mAbs. We speculate that due to the similarity of N-glycan profiles between plant-produced pE60, E16 (Table 1) and h-13F6ΔXF (Zeitlin et al., 2011), WTPε60 and ΔXFPε60 may also have similar preferential binding to C1q and various FcγRs as plant-produced E16, thereby reducing their ability to trigger ADE. Specifically, WTPε60 may have reduced binding to all FcγRs, therefore forgoing its ADE activity. Similar to ΔXFT-produced h-13F6ΔXF and E16, ΔXFPε60 may also have reduced binding to FcγRII and consequently has reduced ADE in K562 cells, but may preserve its binding to C1q as well as to FcγRIII, an FcγR that has been shown not to play a role in the ADE of primary human monocytes. Even though the in vivo effect of ADE reduction by WTpE60 and ΔXFPε60 awaits further confirmation with other FcγR-expressing cell lines and ultimately studies in animal models, our results with K562 cells demonstrate the elimination of ADE mediated by FcγRIIa, the most dominant DENV-mediating and the most widely distributed FcγR subclass expressed on many cell types (Boonnak et al., 2013; Taylor et al., 2015). Although it was previously reported that the aglycosylated aE60 could eliminate the risk of ADE (Balsitis et al., 2010; Williams et al., 2013), the complete removal of N-linked glycans and the resulting total abolishment of binding to C1q and all FcγRs may also render the antibody unstable (Zheng et al., 2011; Kayser et al., 2011) and cause it to lose the necessary effector function for its full therapeutic efficacy (Garcia et al., 2006; Laoprasopwattana et al., 2007; Mehlhop et al., 2009). Even though antibodies against NS1 are the predominant examples that can protect against flaviviruses through ADCC and CDC (Chung et al., 2007; Schlesinger et al., 1987), it has been shown that FcγR-dependent effector function and C1q also play important roles for the function of anti-flavivirus E antibodies (Mehlhop et al., 2009; Oliphant et al., 2005). It was shown that the interaction between C1q and mAbs against WNV E protein greatly enhanced mAb’s in vitro neutralization potency and in vivo efficacy by reducing the stoichiometric threshold of antibodies that are required to neutralize infectivity without the need for other components of the complement (Mehlhop et al., 2009). C1q can also reduce ADE by reducing the threshold of neutralization below the minimal number of antibodies required for ADE (Mehlhop et al., 2009). In contrast to aE60, our plant-derived E60 glycovariant ΔXFPε60 not only minimized ADE in K562 cells, but may retain the potential to bind C1q, suggesting plant-derived proteins may have better efficacy than their aglycosylated counterparts. Collectively, these results suggest the potential of plant-made mAb glycovariants to address ADE and allow the development of effective and safe therapeutics against DENV. This will have important implications for mAb therapeutics beyond the DENV model. For example, ZIKV and DENV are closely related flaviviruses, and antibodies against DENV often cross-react with ZIKV, strongly indicating ADE response may occur between these two viral diseases (Dejnirattisai et al., 2016). Thus, the ability to potentially eliminate ADE by plant-produced mAbs will improve the safety and efficacy of mAb therapeutics against ZIKV and other ADE-prone viruses such as coronaviruses, paramyxoviruses and lentiviruses (Huismans et al., 2009).

The successful production of E60 in plants also helps to address the economic issues of immunotherapeutics. Since the production of plant biomass and plant-derived proteins can be expanded at a commercial scale without the high capital investments of traditional mammalian cell-culture facilities or bioreactors and expensive tissue culture media, the cost of plant-produced biologics can be greatly reduced (Chen &
Davis, 2016). Indeed, recent case studies have confirmed the long-held belief that plant-produced biologics are more cost effective than traditional platforms. Specifically, studies have revealed that plant-based platforms can substantially reduce the production cost of biologics with upstream costs of goods as low as $1.00–2.00 per kilogram of protein (Tusé et al., 2014). Our results indicated that both WTpE60 and ΔXpE60 accumulated efficiently in N. benthamiana leaves with expression levels comparable to other mAbs produced in plants with original mammalian codons (Chen & Lai, 2014b). Since the coding sequence for the HC and LC was not optimized for plant expression, the accumulation levels of pE60 could be increased significantly by sequence optimization (De Muyneck et al., 2010). Results from our laboratory and others have shown that under optimal conditions, mAbs can be produced up to 0.8–4.8 mg (g LFW)−1, a level that is considered to be commercially feasible for mAb manufacturing (Bendandi et al., 2010; Lai et al., 2010). Plant-produced E60 was readily extracted and purified from leaves with a two-step purification process, which has been shown to be scalable and compliant with current Good Manufacturing Practice (cGMP) regulations (Lai & Chen, 2012; Lai et al., 2010, 2012). This downstream process also removed nucleic acids, protein A and endotoxins to levels below the specifications for injectable drugs (He et al., 2014; Lai et al., 2010). Thus, the quick and high-level accumulation of WTpE60 and ΔXpE60 and their facile recovery by a scalable and cGMP-compliant downstream processing scheme indicates the potential of plants as an attractive platform for producing E60 and other novel mAb-based therapeutics with favourable cost, scalability and regulatory compliance. This is significant because even though generating safer therapeutic reagents for DENV is technically possible in mammalian cells, production in plants will markedly enhance cost-efficiency so that it is economically feasible to produce mAb-based therapeutics for the developing world, where the majority of DENV cases exists.

In summary, we demonstrate the development of glycovariants of a mAb that retained the neutralization potency of the original mAb, but reduced its ability to enhance DENV infection in K562 cells. To our knowledge, this is the first report of ADE modulation by mAb N-glycosylation for a disease for which ADE has pathological relevance. This warrants a future in vivo efficacy study in animal models. Together, our results may lead to the generation of safer and more efficacious antibody-based therapeutics against DENV and other ADE-prone viral diseases and contribute to our understanding of how sugar moieties in mAbs regulate Fc-mediated functions.

METHODS

Construction of expression vectors of E60. The coding gene fragments of V1H and V1 of E60 (GenBank accession nos KC254888.1 and KC254888.1) were synthesized and fused to the corresponding DNA sequences of human IgG1 CH1 and CH2 regions, respectively. The resulting coding sequences of E60 HC and LC were cloned into MagnICON-based plant expression vectors pICH21595 and pICH11599 with EcoRI and BamHI restriction enzymes, and transformed into A. tumefaciens as described previously (He et al., 2012; Lai et al., 2010). The E60 LC and HC sequences were also cloned into mammalian cell expression vector pcDNA3.1 (Thermo Fisher Scientific) with BamHI and NotI, and transfected into CHO-K1 cells with lipofectamine (Thermo Fisher Scientific) according to the manufacturer’s protocol. Specifically, 1 µg pcDNA3.1-E60HC and 1 µg pcDNA3.1-E60LC were mixed with 100 µl Opti-MEM media (Thermo Fisher Scientific) and 12.5 µl lipofectamine, and incubated for 45 min at room temperature (RT). After the incubation, the mixture containing the DNA–liposome complexes was diluted with 300 µl Opti-MEM, and 200 µl of the diluted complex mixture was then mixed with 200 µl F12 media (Thermo Fisher Scientific) and overlaid onto 3.5×105 CHO-K1 cells (at approximately 90% confluence) in a 12-well plate. After 5 h of incubation in a CO2 (5%) incubator, serum was added to each well to reach the final concentration of 10%, and cells were incubated for an additional 48 h. Following incubation, cells were treated with trypsin-EDTA (Thermo Fisher Scientific) and 5–10 cells were seeded into a well on a 96-well plate in F12 media containing 250 µg zeocin ml−1 (for HC selection) and 300 µg hygromycin ml−1 (for LC selection) to select transfected cells. Twenty-one days later, positive single-cell colonies were transferred to 48-well plates. Cells were allowed to become nearly confluent and were then transferred into the next larger volume (i.e. 24-well, 12-well and 6-well plates).

Transient expression of pE60 mAb variants in N. benthamiana leaves. WT and ΔXFT N. benthamiana plants were grown and agroinfiltrated with A. tumefaciens strains that contained E60 HC and LC 3’ modules along with their respective 5’ modules and an integrase construct as described previously (Chen & Lai, 2014a; Chen et al., 2013; Leuzinger et al., 2013). To enhance the assembly of pE60 molecules, the OD600 ratio of 4:1 for A. tumefaciens harbouring the HC construct: A. tumefaciens harbouring the LC construct was used for agroinfiltration.

Extraction and purification of pE60 variants from plant leaves. Agroinfiltrated leaves were harvested at 5, 6, 7, 8 and 9 DPI to evaluate the temporal expression pattern of pE60. For all other protein analyses, leaves were harvested at 8 DPI. pE60 variants were extracted and purified with a method developed previously for anti-WNV mAbs (Lai et al., 2010, 2012). Briefly, the crude leaf extract was obtained by homogenization in extraction buffer (50 mM Tris/HCl pH 7.5, 150 mM NaCl) and clarified by centrifugation at 15000 g for 30 min at 4°C. pE60 variants in clarified protein extract were purified by a two-step purification process comprised of ammonium sulfate precipitation followed by protein A affinity chromatography.

Purification of mE60 from CHO cell culture. The culture fluid from the E60-transfected cell line was clarified by spinning at 15000 g for 30 min at 4°C. mE60 in clarified media was purified directly by protein A affinity chromatography as described previously (Lai et al., 2012).

Gel electrophoresis, Western blot and flow cytometry with yeast surface display. Electrophoresis was performed either with 10% SDS-PAGE under reducing (5%, v/v, β-mercaptoethanol) or 4–20% gradient SDS-PAGE under non-reducing conditions. Gels were stained with Coomassie blue or used to transfer proteins onto PVDF membranes. HRP-conjugated antibodies against human-kappa LC or gamma HC (Southern Biotech) were used for Western blot analysis as described previously (Lai et al., 2014). p60 variant expression was examined by ELISA that detected the assembled form of mAbs with both HC and LC, as described (Lai et al., 2010). Briefly, plates were coated with a goat anti-human gamma HC antibody (Southern Biotech). After incubation with the plant protein extract, a HRP-conjugated anti-human kappa LC antibody was used for detection. A plant-produced mAb with human IgG1 CH1 and kappa Ck (E16) (Lai et al., 2010) was used as a reference standard. Yeast cells displaying DENV EDE-EDII on their surface were stained with mAb variants, and analysed with a Becton Dickinson FACS Calibur flow cytometer as described previously (Lai et al., 2010).

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ELISA. The ELISA to examine the binding of pE60 to DENV-2 E protein was performed as described previously (He et al., 2012). DENV-2 E (Creative Diagnostics) was coated on microtitre plates. A HRP-conjugated anti-gamma-HC antibody was used as the detection antibody. mE60 and a generic human IgG were used as a positive and a negative control, respectively. The plates were developed with tetramethylbenzidine substrate (KPL). Experiments were performed three times with technical triplicates for each sample. The binding data were analysed with GraphPad Prism software. $K_d$ was determined by non-linear regression analysis using a one-site binding model. The $K_d$ of pE60 was compared with that of mE60, and $P$ values were determined by unpaired t-test. A $P$ value of $<0.05$ indicated statistically significant differences.

N-linked glycan analysis. The N-linked glycosylation profiles of E60 variants were determined by LC–ESI–MS, as reported previously (Stadlmann et al., 2008). Briefly, purified E60 variants were separated by 10% SDS-PAGE under reducing conditions. After detection with Coomassie stain, the HC-containing band was excised from the gel. Peptide fragments were eluted from the gel with 50% acetonitrile after S-alkylation and tryptic or tryptic/GluC digestion and separated on a reversed-phase column (150×0.32 mm BioBasic-18, Thermo Fisher Scientific) with a 1–80% acetonitrile gradient. A quadruple time-of-flight (Q-TOF) Ultima Global mass spectrometer (Waters) was used to analyse the glycopeptides. Spectra were summed and deconvoluted for identification of glycoforms. The ProGlyCAn nomenclature (www.proglycan.com) was used to annotate the glycans.

**Antibody-dependent enhancement assay.** The enhancing activities of the mAbs were determined with FcγRIIA$^+$ K562 cells (ATCC CCL-2243). Eight three-fold serial dilutions of E60 variants or a generic human IgG (negative control) were incubated with DENV-2 (ATCC VR-1584) for 1 h at 37°C and subsequently mixed with K562 cells (m.o.i. =1). After 48 h of incubation, cells were washed with PBS, fixed with 4% paraformaldehyde (Sigma) and permeabilized with 0.1% saponin (Sigma). Permeabalized cells were then stained with 4G2 (ATCC HB112) conjugated to Alexa 488 (Invitrogen), washed and analysed with a Navios flow cytometer (Beckman Coulter) to determine the percentage of infected (4G2-positive) cells.

**DENV-2 and DENV-4 neutralization.** MAbs were serially diluted in serum-free medium (SFM; Opti-MEM serum-free medium, Life Technologies), while DENV-2 (ATCC VR-1584) and DENV-4 (TUP-2174, kindly provided by Dr John F. Anderson) were diluted in SFM to a working concentration of 100 p.f.u. per well. Following dilutions, DENV-2 or DENV-4 were added to the diluted mAbs and incubated for 1 h at RT. Following incubation, DENV–mAb mixtures were added to a 90–95% confluence of Vero cells (ATCC CCL-81, 1×10$^6$ cells ml$^{-1}$) in SFM and were incubated for 1 h at 37°C. Medium containing virus–mAbs was removed and overlaid with fresh medium (OptiMEM-Glutamax containing 2% FBS and 1% methicellulose, Sigma) and incubated for an additional 4 days at 37°C. Following incubation, an immunostaining assay was performed as described previously (Paul et al., 2014). Briefly, after the overlay medium was removed, the cells were washed twice with PBS and fixed in 4% paraformaldehyde (PFA). The cells were washed and blocked with 2% normal goat serum (Life Technologies) with 0.4% Triton-X100 (Sigma) for 1 h at RT, then probed with monoclonal mouse anti-flavivirus E IgG antibody 4G2 (ATCC D1-4G2-4–15 HB-112; 1:100 dilution) overnight at 4°C. The cells were washed in PBS, and polyclonal goat anti-mouse-HRP second antibody IgG (KPL; 1:1000) was added for 2 h at RT. Immunopositive foci were detected with TrueBlue peroxidase substrate (KPL) and counted as f.f.u. using an Axioscop Plus light microscope (Zeiss). Percentage (%) neutralization was calculated as: [(number of DENV f.f.u. per well with no mAb)–(number of DENV f.f.u. per well of diluted mAb)/(number of DENV f.f.u. per well with no mAb)×100]. The half-maximal effective concentration (EC$_{50}$) of each mAb was calculated using GraphPad Prism software (version 6.0).

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