In vitro and in vivo efficacy of anti-chikungunya virus monoclonal antibodies produced in wild-type and glycoengineered *Nicotiana benthamiana* plants

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Summary

Chikungunya virus (CHIKV) is a mosquito-transmitted alphavirus, and its infection can cause long-term debilitating arthritis in humans. Currently, there are no licensed vaccines or therapeutics for human use to combat CHIKV infections. In this study, we explored the feasibility of using an anti-CHIKV monoclonal antibody (mAb) produced in wild-type (WT) and glycoengineered (ΔXFT) *Nicotiana benthamiana* plants in treating CHIKV infection in a mouse model. CHIKV mAb was efficiently expressed and assembled in plant leaves and enriched to homogeneity by a simple purification scheme. While mAb produced in ΔXFT carried a single N-glycan species at the Fc domain, namely GnGn structures, WT produced mAb exhibited a mixture of N-glycans including the typical plant $GnGnXF_3$ glycans, accompanied by incompletely processed and oligomannosidic structures. Both WT and ΔXFT plant-produced mAbs demonstrated potent *in vitro* neutralization activity against CHIKV. Notably, both mAb glycoforms showed *in vivo* efficacy in a mouse model, with a slight increased efficacy by the ΔXFT-produced mAbs. This is the first report of the efficacy of plant-produced mAbs against CHIKV, which demonstrates the ability of using plants as an effective platform for production of functionally active CHIKV mAbs and implies optimization of *in vivo* activity by controlling Fc glycosylation.

Introduction

Chikungunya virus (CHIKV) is a positive-sense RNA virus belonging to the alphavirus family, which can be transmitted to humans by mosquitoes. The viral RNA is encapsulated in a central capsid core, which, in turn, is surrounded by the viral envelope studded with viral envelope proteins 1 and 2 (E1 and E2) (Voss *et al.*, 2010). Infection of CHIKV in humans can cause debilitating polyarthralgia that may persist months to years and affects multiple joints including ankles, knees, wrists and fingers (Schilte *et al.*, 2013). Since 2004, CHIKV reemergence has resulted in millions of cases of severe and often chronic arthralgia on five continents (Weaver and Forrester, 2015). Currently, there are no licensed therapeutics or vaccines against CHIKV for human use. Continued outbreaks of CHIKV and the risk of it spreading into new areas call for the urgent development of therapeutics to treat this debilitating virus.

The potential utility of antibodies as efficacious anti-CHIKV therapeutics was suggested by early vaccine studies. It was found that vaccine potency correlates with its ability of inducing neutralizing antibodies in mice. Neutralizing antibodies from past infections in humans also show evidence of protectivity, as these subjects are immune to CHIKV reinfection (Kam *et al.*, 2012; Yoon *et al.*, 2015). Indeed, monoclonal antibodies (mAbs) against CHIKV E1 and E2 have been shown to be protective against CHIKV infection in various mouse models. For example, anti-E1 or E2 mAbs protected immune compromised (Ifnar -/-) mice against lethal challenges of CHIKV when given as post-exposure therapy

(Pal *et al.*, 2013). Interestingly, it was also revealed that in addition to neutralizing potency, antibody Fc effector functions also contribute to the efficacy of anti-CHIKV E1 and E2 antibodies (Pal *et al.*, 2013).

Plants have been demonstrated to be an attractive alternative to mammalian cell cultures as a system for mAb development and production (Chen and Davis, 2016). mAbs produced in *Nicotiana benthamiana* have been developed as candidates to treat various viral infections including those caused by West Nile virus (WNV) (Sun *et al.*, 2018), dengue virus (DENV) (Dent *et al.*, 2016) and, of prominence, using a cocktail of three mAbs to treat Ebola virus-infected human patients (PREVAIL Group, 2016). For example, we have demonstrated that a single dose of an anti-WNV mAb produced in *N. benthamiana* and lettuce plants protected mice from lethal infection of WNV, even given 4 days post-infection (dpi) when virus already circulates in the brain (He *et al.*, 2014; Lai *et al.*, 2010, 2012, 2014).

One of the advantages of using plants for the production of glycoproteins is that they are highly amenable to glycoengineering, which allows the generation of proteins with tailor-made N-glycans (Montero-Morales and Steinkellner, 2018). This is particularly relevant to mAbs since it is well known that the glycosylation profile on the single N-glycosylation site in the Fc domain of IgG antibodies significantly impacts mAb activities (Jefferis, 2012). For example, the targeted removal of core fucose residues from complex N-glycans in mammalian and plant-produced IgGs significantly increases Fc gamma receptor (FcγR) III binding and subsequent effector functions. This has been demonstrated

impressively for anti-cancer antibodies, where the first generation of glycan-engineered antibody therapeutics is ready to emerge on the market (Garber, 2018; Reichert, 2012). However, related studies for antibodies against infectious diseases are rare (Forthal *et al.*, 2010), with only a few *in vivo* studies demonstrating increased antiviral activity of glycan-engineered mAbs in murine challenging models (Hiatt *et al.*, 2014; Zeitlin *et al.*, 2011). Nevertheless, the impact of glycosylation to antiviral antibody-induced effector functions is still under debate, as non-human primate studies of anti-HIV antibodies did not show glycan-dependent efficacy (Moldt *et al.*, 2012).

Here, we aimed to produce two glycoforms of anti-CHIKV E1 mAb (CHKVmab) in plants and investigate their *in vitro* and *in vivo* efficacy. CHKVmabs were efficiently expressed and assembled in wild-type (WT) *N. benthamiana*, as well as in ΔXFT, a *N. benthamiana* glycosylation mutant lacking plant-specific core xylose and fucose. ΔXFT-derived CHKVmab (ΔXFpCHKVmab) exhibited mammalian-type GnGn glycans with high uniformity, while WT-produced CHKVmab (WTpCHKVmab) carried a mixture of N-glycans, including typical plant GnGnXF$_3$, incompletely processed and oligomannosidic structures. Both mAb variants potently neutralized CHIKV *in vitro*. Notably, both pCHKVmab glycoforms also showed efficacy but with different potency against CHIKV infection in a murine model.

Results

Expression and assembly of CHKVmab in WT and ΔXF *N. benthamiana* plants

The DNA expression cassette of the heavy chain (HC) and light chain (LC) of CHKVmab was cloned into a MagnICON-based plant expression vector (Giritch *et al.*, 2006), transformed into *Agrobacterium tumefaciens* and agroinfiltrated into WT and ΔXFT *N. benthamiana* leaves (Chen and Lai, 2014a; Leuzinger *et al.*, 2013). The expression of CHKVmab was evaluated by Western blot analysis. As shown in Figure 1, WTpCHKVmab and ΔXFpCHKVmab have HC and LC with the expected molecular weight (Figure 1a,b, Lanes 3–4), and are fully assembled into the heterotetramer equivalent to a positive control IgG (Figure 1c, Lanes 3–4). The expression level of WTpCHKVmab and ΔXFpCHKVmab was estimated to be 100 and 130 μg/g leaf fresh weight (LFW), respectively. This level of expression is similar to that previously reported for non-codon-optimized mAbs produced in plants (De Muynck *et al.*, 2010; Dent *et al.*, 2016; Lai *et al.*, 2010).

Purification of CHKVmab from *N. benthamiana* leaves

We have previously developed a two-step extraction and purification process consisting of low pH precipitation and protein A chromatography for mAbs produced in plants (Lai *et al.*, 2010, 2012). Using this method, CHKVmab was purified from *N. benthamiana* leaves to >90% homogeneity (Figure 2). Purified mAbs were used for further biochemical and functional analyses.

N-linked glycosylation profiles of plant-produced CHKVmabs

Since the structure of N-glycans in the Fc region of an antibody affects its Fc-mediated effector functions (Jefferis, 2012), the N-glycosylation of pCHKVmabs was determined by liquid chromatography–electrospray ionization–mass spectrometry (LC-ESI-MS). pCHKVmab produced in ΔXFT plants exhibited the expected

mammalian-type GnGn N-glycan structure lacking plant-specific xylose and fucose with a high degree of uniformity (>95%) (Table 1). Glycosylation pattern of WTpCHKVmab was found to carry a mixture of N-glycans (Table 1): the major form was as the expected plant typical GnGnXF$_3$ structure (33%), accompanied by incompletely processed complex N-glycans (39%) and oligo-mannosidic structures (28%).

Neutralization activity of plant-derived CHKVmab against CHIKV

Plaque reduction neutralization test (PRNT) was used to evaluate the neutralization potential of plant-produced mAbs against CHIKV (Yang *et al.*, 2017b). While the negative control mAb (an anti-WNV mAb) did not neutralize CHIKV, both WTpCHKVmab and ΔXFpCHKVmab showed strong neutralizing activity against CHIKV with statistically similar potency (ΔXFpCHKVmab EC$_{50}$ = 130.5 ng/mL vs. WTpCHKVmab EC$_{50}$ = 390.8 ng/mL; P = 0.33) (Figure 3). The mean EC$_{50}$ values of plant-derived CHKVmabs are comparable to those of mammalian cell-produced orthologue (mCHKVmab EC$_{50}$ = 154 ng/mL) (Pal *et al.*, 2013). Overall, WTpCHKVmab and ΔXFpCHKVmab exhibited potent neutralizing activity *in vitro* against infectious CHIKV.

In vivo efficacy of plant-derived CHKVmab against CHIKV infection

Among mouse models of CHIKV infection, WT mice (i.e. WT C57BL/6) develop arthritis symptoms similar to those observed in humans (Broeckel *et al.*, 2017). Thus, we used the WT C57BL/6 mouse model to assess the *in vivo* therapeutic efficacy of plant-produced CHKVmabs (Acharya *et al.*, 2015). Five-week-old C57BL/6 mice were inoculated in the right footpad with 1 × 10^5 PFU of CHIKV and then treated at 12 h post-infection with 50 μg of WTpCHKVmab, ΔXFpCHKVmab or PBS intraperi-toneally. Since viraemia generally peaks at 2 dpi and subsides to undetectable levels at 4 dpi in this mouse model, blood was collected at 2 dpi and viraemia measured by RT-qPCR. Mice treated with both WTpCHKVmab and ΔXFpCHKVmab developed significantly lower viraemia compared to PBS-treated mice (P < 0.05 for WTpCHKVmab; P < 0.01 for ΔXFpCHKVmab) (Figure 4). Interestingly, ΔXFpCHKVmab appeared to be more potent than WTpCHKVmab in reducing viral load in mice (Figure 4). Statistical analysis indicated that albeit not dramatic, the difference in efficacy between the two mAb glycovariants was nonetheless significant (P < 0.05, ΔXFpCHKVmab compared to WTpCHKVmab). Collectively, the *in vivo* efficacy of plant-derived CHKVmabs in WT mice suggests their potential as prophylaxis and post-exposure therapeutics for CHIKV.

Antibody-dependent enhancement activity of plant-produced CHKVmab for DENV infection

One of the challenges for mAb therapeutics against viral infections is the increased risk of infection by other viruses in treated patients via the mechanism of antibody-dependent enhancement of infection (ADE). For example, cross-reactive but sub-neutralizing antibodies against one serotype of DENV from a previous infection can form complexes with another serotype of DENV during a secondary infection to promote viral infection of FcγR-bearing myeloid cells, predisposing patients to develop the more severe dengue haemorrhagic fever/dengue shock syndrome through ADE (Sun *et al.*, 2018). Since the E proteins of CHIKV and DENV are related and the two viruses co-circulate geographically (Kam *et al.*, 2015), we examined
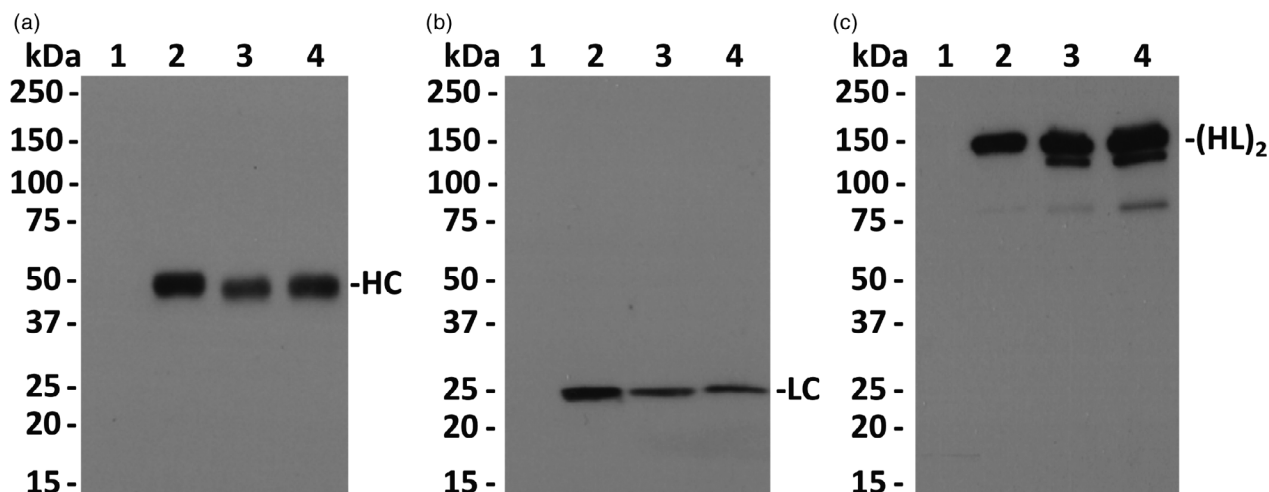
Figure 1 Western blot analysis of plant-produced CHKVmab. CHKVmab was extracted from WT or ΔXFT *Nicotiana benthamiana* leaves, separated on SDS-PAGE gels under reducing (a and b) or non-reducing (c) conditions, and blotted onto PVDF membranes. A goat anti-human gamma chain antibody (a) or a goat anti-human kappa chain antibody (b and c) was incubated with the membranes to detect heavy chain or light chain. Lane 1, protein sample extracted from non-infiltrated leaves; Lane 2, mammalian cell-produced anti-WNV E16 mAb as a reference standard; Lane 3, sample from leaves of WT plants infiltrated with CHKVmab constructs; Lane 4, sample from leaves of ΔXFT plants infiltrated with CHKVmab constructs. HC: heavy chain, LC: light chain, (HL)$_2$: assembled mAb with two light and heavy chains.
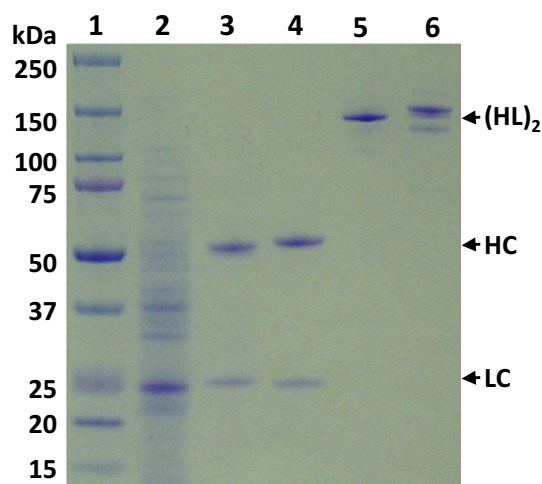

Figure 2 Purification of CHKVmab from *Nicotiana benthamiana* leaves. Total soluble proteins were extracted from plants, and CHKVmab was purified and analysed on a 4%–20% gradient SDS-PAGE gel under reducing (Lanes 1–4) or non-reducing (Lanes 5 and 6) conditions and visualized with Coomassie stain. Lane 1, protein molecular weight marker; Lane 2, leaf soluble proteins after low pH precipitation; Lanes 3 and 5, CHKVmab purified from leaves; Lanes 4 and 6, mammalian cell-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.

Table 1 N-linked glycans of CHKV mAb variants

Sample	GnGnXF$_3$	GnGnX	GnMXF$_3$	GnGn	Man$_{(8-9)}$
ΔXFpCHKV				>95	
WTpCHKV	33	15	9	15	28

N-glycosylation profile as determined by liquid chromatography–electrospray ionization–mass spectrometry (LS-ESI-MS). Numbers represent the presence of various glycoforms in percentage. Note ΔXFT is a *Nicotiana benthamiana* N-glycosylation mutant that lacks plant-specific core xylose and fucose. Glycans were annotated according to the ProGlycAn nomenclature (www.proglycan.com).

Discussion

The expanding epidemics of CHIKV demand the development of efficacious therapeutics against this debilitating virus. A screening of 230 mouse anti-CHIKV mAbs indicate that some of the antibodies against CHIKV E1 and E2 have protective activity and their protectivity is mostly correlated to the neutralizing potency (Pal *et al.*, 2013). Furthermore, antibody Fc effector functions have been found to be crucial for the efficacy of these anti-CHIKV antibodies (Pal *et al.*, 2013). In the current study, we explored the feasibility of using plant-produced mAbs against E1 in treating CHIKV infection in a mouse model as a step towards improving the efficacy of CHIKV immunotherapy in humans.

A prerequisite for plant-made mAbs to be competitive alternatives of those made in mammalian cells is their robust production in plants. While mAb production in mammalian cell culture is highly efficient, production costs are immense ($80 000–160 000/kg protein; Shevitz *et al.*, 2011) and, in many cases, unaffordable to people in many parts of the world. In contrast, production of plant-derived proteins does not require expensive cell-culture facilities, bioreactors, or tissue culture media and production can be scaled up for commercial
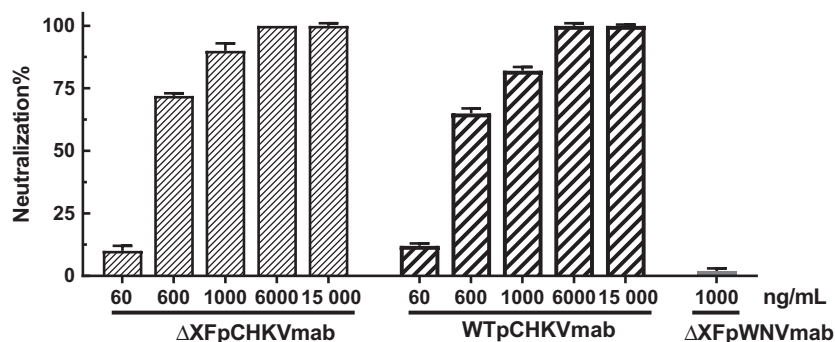
whether CHKVmab would enhance the infection of DENV. As previously demonstrated (Lai *et al.*, 2018), 4G2, an mAb that cross-reacts with E of most flaviviruses, efficiently caused ADE of DENV infection in K562 cells that express the human FcγR (Figure 5). In contrast, ΔXFpCHKVmab and WTpCHKVmab did not promote ADE for DENV (Figure 5). These results indicate that plant-produced CHKVmabs are not only efficacious but also safe as potential treatment against CHIKV infection.

Figure 3 The neutralizing activity of WTpCHKVmab and ΔXFpCHKVmab. Dilutions of WTpCHKVmab, ΔXFpCHKVmab or a ΔXF plant-produced anti-WNV E16 mAb (ΔXFpWNVmab, negative control) were co-incubated with 100 PFU of CHIKV and Vero cells for 3 days. Plaques were counted, and per cent neutralization and EC_{50} were calculated. Experiments were performed twice with technical triplicates for each sample. Bars represent the standard deviation (SD) of the mean.
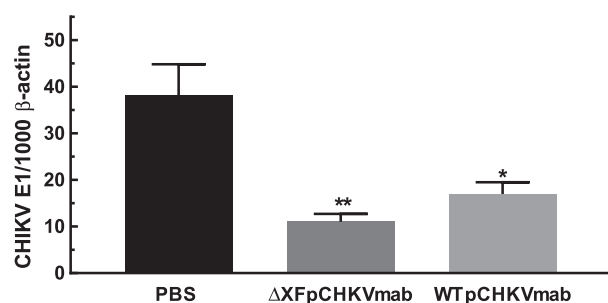

Figure 4 Plant-produced CHKVmabs reduce CHIKV viraemia in mice. Wild-type C57BL/6J mice ($N = 6$ per group) were subcutaneously infected with 1×10^5 PFUs of CHIKV and treated intraperitoneally with 50 μg of indicated CHKVmab or PBS (negative control) 12 h post-infection. Viral load in blood at day 2 dpi is presented as a ratio of CHIKV E1 copy number per 1000 copy of cellular β-actin. ** and * indicate P values <0.01 and <0.05, respectively, of CHKVmab-treated mice compared to that of PBS-treated, which were determined by t-tests.
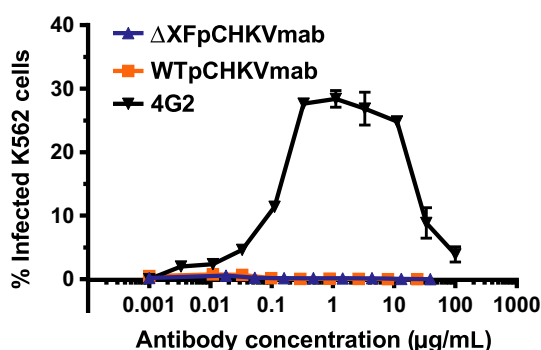

Figure 5 Antibody-dependent enhancement of CHKVmabs for DENV infection. Serial dilutions of CHKVmabs or 4G2 (positive control mAb) were mixed with DENV-2 and then incubated with FcγRIIa+ K562 cells. Cells were fixed, permeabilized and stained with an anti-DENV E antibody and analysed by flow cytometry.

manufacture without prohibitive capital investments (Chen and Davis, 2016; Tuse *et al.*, 2014). Indeed, recent case studies have confirmed that *N. benthamiana*-based platforms can substantially reduce the upstream production cost of biologics to as low as

$1.00–2.00 per kilogram of protein (Tuse *et al.*, 2014). Our results indicated that both WTpCHKVmab and ΔXFpCHKVmab accumulated in *N. benthamiana* leaves to levels comparable to other mAbs produced in plants with non-optimized codons (Chen and Lai, 2014b). Under optimized conditions, however, mAbs can be produced up to 0.8–4.8 mg/g LFW, a commercially feasible level for mAb manufacturing (Bendandi *et al.*, 2010; Lai *et al.*, 2010). Our results also showed that WTpCHKVmab and ΔXFpCHKVmab were readily extracted and purified from leaves with a scalable process that is compliant to current Good Manufacturing Practice (cGMP) regulations (Lai and Chen, 2012; Lai *et al.*, 2010). In fact, biotechnology companies, such as Fraunhofer IME, already have a cGMP manufacturing licence for plant-produced mAbs (Ma *et al.*, 2015) and can easily adopt the downstream process we developed here for the future manufacturing of CHKVmabs. Together, these results indicate that similar to CHIKV vaccine development (Cardona-Ospina *et al.*, 2016; Salazar-Gonzalez *et al.*, 2015), the *N. benthamiana*-based plant system has the potential to produce anti-CHIKV mAbs with favourable yield and cost, which will markedly increase the affordability of mAb-based CHIKV drugs to people in the developing world, where the majority of CHIKV cases occurs.

Another striking feature of the plant transient expression platform is the rapid production for an identified target. This is particularly important in cases of sudden disease outbreaks and the subsequent urgent need of therapeutics for immediate use, a typical scenario of viral outbreaks. In the plant transient system, a time frame of 1–2 weeks is sufficient for the generation of 1 g of product per kg of leaves after gene construct delivery (Lomonossoff and D'Aoust, 2016). Based on our data and information available from ZMapp large-scale production (Arntzen, 2015; Holtz *et al.*, 2015; Lai and Chen, 2012; Zeitlin and Haydon, 2015), we estimate that a current U.S.-based contract manufacturing facility (CMO) can generate leaves containing 1 kg of CHKVmab within 2 weeks from the delivery of the expression vectors described in this study. At this moment, this production speed cannot be matched by any other expression system and our work may contribute to rapidly producing pharmaceuticals to combat emerging diseases.

To be considered as therapeutic candidates, plant-made mAbs must have at least equivalent potency as their mammalian cell-produced counterparts. A major mechanism of antiviral activity

for anti-CHIKV mAbs is viral neutralization (Pal *et al.*, 2013). Plant-made CHKVmab exhibited strong neutralizing activity against CHIKV with potencies similar to mammalian cell-derived CHKVmab (Pal *et al.*, 2013). Importantly, *in vitro* activities of plant-made CHKVmabs translated to *in vivo* efficacy. There are several mouse models for evaluating the protectivity of mAbs against CHIKV. Immune compromised (Ifnar -/- or AG129) mice are subject to lethal CHIKV infection, providing a convenient survival model (Pal *et al.*, 2013). However, these mice do not develop the arthritis observed in humans. In contrast, CHIKV infection in WT C57BL/6 mice causes arthritis and swelling in feet and ankles (Broeckel *et al.*, 2017), presenting a more relevant animal model for studying human CHIKV infection. CHIKV infection in WT C57BL/6 mice does not cause lethal infection, but a transient viraemia – detectable at 1 dpi, reaching its peak at 2 dpi and subsiding by 4 dpi (Acharya *et al.*, 2015). In this relevant model, we observed that pCHKVmabs significantly reduced titres of virus in blood at 2 dpi, demonstrating the potential of plant-produced mAbs as efficacious post-exposure therapeutics *in vivo*.

One of the advantages of using plant-based platforms to produce antibodies is the ability to alter N-glycans present on the single glycosylation site at the Fc domain (Strasser *et al.*, 2014). IgG Fc effector functions depend on the specific interaction between the IgG Fc domain and FcγR on immune cells which, in turn, is highly sensitive to the composition of N-linked Fc glycans (Jefferis, 2012). MAbs produced in mammalian cells usually carry a mixture of N-glycans, with some glycoforms being more bioactive than others (Jefferis, 2005). As such, glycoengineered plant lines that produce mAbs with distinct N-glycans are highly appreciated for optimizing the therapeutic potency of mAbs, especially for those that require Fc effector function for efficacy. For example, glycoengineered plants have been used to increase the efficacy of anti-filovirus and anti-HIV antibodies (Forthal *et al.*, 2010; Zeitlin *et al.*, 2011). Moreover, our laboratory has demonstrated that anti-DENV antibodies produced in ΔXFT and WT *N. benthamiana* plants forgo their ADE activity and, consequently, have superior efficacy and safety profiles than their mammalian-produced counterparts most likely due to their specific N-glycosylation profile (Dent *et al.*, 2016). Since anti-CHIKV E1 and E2 mAbs require Fc effector function for their efficacy (Pal *et al.*, 2013), their activities may be optimized by modulating glycosylation profiles. Indeed, our results showed that ΔXFpCHKVmab had better *in vivo* efficacy against CHIKV than WTpCHKVmab. These results support our hypothesis that some antibody glycoforms may have superior protectivity than others by enhancing antibody's effector functions. We believe that the difference in potency between ΔXFpCHKVmab and WTpCHKVmab can be much greater if they both carry a homogenous glycoform. These results warrant further studies with additional anti-CHIKV mAbs using multiple glycoengineered plant lines (Chen, 2016; Montero-Morales and Steinkellner, 2018).

Collectively, we demonstrate the efficient expression, purification and the potent efficacy of two glycoforms of an anti-CHIKV E1 mAb in plants and show that their *in vivo* potency may be optimized by the attachment of defined glycan profiles. In addition, neither of the two mAb glycoforms promoted ADE for DENV infection. These results suggest that mAbs have the potential to be used as effective and safe prophylaxis to prevent CHIKV infection during outbreaks, especially for populations with high risk of developing severe diseases. To our knowledge, this is the first report of the efficacy of plant-produced mAbs against CHIKV. Our results warrant future studies in mouse arthritis models that mimic symptoms of human CHIKV infection. In the context of recent studies that demonstrate broadly neutralizing human mAbs against CHIKV E2 with therapeutic activity (Smith *et al.*, 2015), our work can be expanded to the development of a novel multi-target therapy to combat sudden CHIKV outbreaks.

Materials and methods

Ethics statement and biosafety

All animal experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committees (IACUC) at the University of Southern Mississippi (USM). All *in vitro* experiments and animal studies involving live CHIKV were performed by the certified personnel in the USM biosafety level 3 (BSL3) facility by following standard biosafety protocols approved by the USM Institutional Biosafety Committee (IBC).

Vector construction for expression of CHKVmab

The coding sequence of CHKVmab variable region of HC (V_H) and LC (V_L) (Pal *et al.*, 2013) was fused to the corresponding DNA sequences of human IgG1 constant regions of HC (C_H) and LC (C_L), respectively. The resulting HC and LC coding sequences were cloned into MagnICON-based plant expression vectors pICH21595 and pICH11599, and transformed into *A. tumefaciens* as described previously (He *et al.*, 2012; Lai *et al.*, 2010).

Transient expression of pCHKVmab in *N. benthamiana* leaves

Wild-type and ΔXFT *N. benthamiana* plants were grown and agroinfiltrated with *A. tumefaciens* strains that contain CHKVmab HC and LC 3′ modules along with their respective 5′ modules, and an integrase construct as described previously (Chen *et al.*, 2013; Leuzinger *et al.*, 2013).

Extraction and purification of pCHKVmab from plants

Agroinfiltrated leaves were harvested 7 dpi, and CHKVmab was extracted and purified with a method previously developed for anti-WNV and DENV mAbs (Dent *et al.*, 2016; Lai *et al.*, 2010, 2012). Briefly, the crude leaf extract was obtained by homogenization in extraction buffer (1 × PBS pH 5.2, 10 mg/mL sodium ascorbate, 1 mM EDTA) and clarified by centrifugation at 15 000 ***g*** for 30 min at 4 °C. CHKVmab in clarified protein extract was purified by a two-step purification process comprised of low pH precipitation followed by protein A affinity chromatography.

Protein analyses

SDS-PAGE (10% or 4%–20% gradient) was performed either under reducing (5% v/v β-mercaptoethanol) or non-reducing conditions. SDS-PAGE gels were stained with Coomassie blue. For Western blot analysis, proteins on SDS-PAGE gels were transferred onto PVDF membranes and detected with horseradish peroxidase (HRP)-conjugated antibodies against human kappa LC or gamma HC (Southern Biotech, Birmingham, AL) as previously described (Lai *et al.*, 2014).

The expression level of CHKVmab was measured by an ELISA that detected the fully assembled form of mAbs with both HC and LC (Lai *et al.*, 2010). Briefly, plates were coated with an anti-human gamma HC antibody (Southern Biotech) and incubated with the plant protein extract. After washing, a HRP-conjugated anti-human

kappa LC antibody (Southern Biotech) was used for detection. A plant-produced mAb with human IgG1 CH and kappa CL (E16) (Lai *et al*., 2010) was used as a reference standard.

N-linked glycan analysis

LC-ESI-MS was used to determine the N-linked glycosylation profiles of CHKVmab variants as previously reported (Dent *et al*., 2016). Briefly, the HC band of purified CHKVmabs on Coomassie-stained SDS-PAGE was excised from the gel. Peptide fragments were generated by S-alkylation and tryptic or tryptic/GluC digestion and subsequently eluted from the gel with 50% acetonitrile. Peptide fragments were then separated on a Reversed-Phase Column (150 × 0.32 mm BioBasic 18, Thermo Fisher Scientific) with a 1%–80% acetonitrile gradient. Glycopeptides were analysed with a quadruple time-of-flight (Q-TOF) Ultima Global mass spectrometer (Waters, Milford, MA). Spectra were summed and deconvoluted for identification of glycoforms. The ProGlycAn nomenclature (www.proglycan.com) was used to annotate the glycans.

Virus and cells

CHIKV (strain LR-OPY1, kindly provided by Dr. Robert B. Tesh at University of Texas Medical Branch) was propagated and tittered in Vero cells (ATCC, CCL-81). Vero cells were cultured in DMEM supplemented with 10% FBS at 37 °C in an incubation with 5% CO_2.

CHIKV neutralization and plaque assay

Plaque reduction neutralization test (PRNT) was performed as previously described (Yang *et al*., 2017a, 2018). Specifically, mAbs were serially diluted in phosphate-buffered saline (PBS), while CHIKV was diluted in serum-free DMEM to a working concentration of 100 plaque-forming units (PFUs) per well. Following dilutions, mAbs were added to CHIKV and incubated for 1 h at 37 °C. CHIKV-mAb mixes were then added to a 90%–95% confluence Vero cells in a 6-well tissue culture plate and incubated for 1 h at 37 °C for virus attachment. After removing unattached virus-mAbs in the medium, cells were overlaid with fresh media (complete DMEM containing 1% sea plaque agarose, Lonza) and incubated for an additional 3 days at 37 °C. Plaques were visualized by staining with neutral red (Sigma) and counted. Percent (%) neutralization was calculated as: [(number of CHIKV plaque per well with no mAb) − (number of CHIKV plaque per well of diluted mAb)/(number of CHIKV plaque per well with no mAb) × 100]. Experiments were repeated twice. The half maximal effective concentration (EC_{50}) of each mAb was calculated using GraphPad Prism software (version 6.0).

Animal studies

Female C57BL/6J mice (5 weeks old, six mice per group) were purchased from the Jackson Laboratory and subcutaneously inoculated on the ventral side of the right hind footpad with 10^5 PFUs of CHIKV as previously described (Acharya *et al*., 2015). Mice were intraperitoneally inoculated with 50 μg of CHKVmabs or PBS at 12 h post-CHIKV infection. Mice were bled retro-orbitally at 2 dpi to measure viraemia.

Real-time quantitative PCR (RT-qPCR)

Total RNA was isolated from mouse blood samples using TRI reagent (Molecular Research Center, Inc.). The first-strand complementary DNA (cDNA) was synthesized by using the iSCRIPT cDNA synthesis kit (Bio-Rad). CHIKV envelope protein 1 (CHIKV

E1) and cellular β-actin RNA copy numbers were determined by RT-qPCR in a CFX96 Real-Time System (Bio-Rad) using SYBR Green Supermix (Bio-Rad). Viral copy numbers were expressed as the ratio of CHIKV E1 to cellular β-actin. Primer sequences for mouse β-actin and CHIKV E1 gene were previously described (Acharya *et al*., 2015).

Antibody-dependent enhancement assay

The potential enhancing activities of the anti-CHIKV mAbs for DENV infection were examined by using FcγRIIa$^+$ K562 cells (ATCC, CCL-2243) as previously described (Dent *et al*., 2016). Serial dilutions of CHKVmabs or the positive control mAb 4G2 were initially incubated with DENV-2 (ATCC, VR-1584) for 1 hr at 37 °C. Thereafter, the mAb–virus complexes were incubated with K562 cells (MOI = 1) for 48 h. Cells were then fixed with 4% paraformaldehyde (Sigma), permeabilized with 0.1% saponin (Sigma), and stained with Alexa 488 (Invitrogen)-conjugated 4G2 (ATCC, HB112). Stained cells were washed and analysed with a Navios flow cytometer (Beckman Coulter) to determine the percentage of infected cells.

Statistical analyses

Data analysis was performed using GraphPad Prism software version 6.0 (GraphPad, CA). Comparisons of neutralization potency between different mAbs were performed using *t*-tests. Comparison of blood viral copy numbers between mouse groups treated with different mAbs was also performed by *t*-tests. A *P* value of <0.05 indicated statistically significant difference.

Author contributions

Q.C. designed the research; J.H., D.A., H.L., H.S., and S.K. performed the research; J.H., D.A., H.L., H.S., F.B. and Q.C. analysed the data; and Q.C. wrote the paper with H.S. and F.B. providing critical comments.

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References

Acharya, D., Paul, A.M., Anderson, J.F., Huang, F. and Bai, F. (2015) Loss of glycosaminoglycan receptor binding after mosquito cell passage reduces chikungunya virus infectivity. *PLoS Negl. Trop Dis.* **9**, e0004139.

Arntzen, C. (2015) Plant-made pharmaceuticals: from 'Edible Vaccines' to Ebola therapeutics. *Plant Biotechnol. J.* **13**, 1013–1016.

Bendandi, M., Marillonnet, S., Kandzia, R., Thieme, F., Nickstadt, A., Herz, S., Frode, R. *et al*. (2010) Rapid, high-yield production in plants of individualized idiotype vaccines for non-Hodgkin's lymphoma. *Ann. Oncol.* **21**, 2420–2427.

Broeckel, R., Fox, J.M., Haese, N., Kreklywich, C.N., Sukulpovi-Petty, S., Legasse, A., Smith, P.P. *et al*. (2017) Therapeutic administration of a recombinant human monoclonal antibody reduces the severity of chikungunya virus disease in rhesus macaques. *PLoS Negl. Trop Dis.* **11**, e0005637.

Cardona-Ospina, J.A., Sepulveda-Arias, J.C., Mancilla, L. and Gutierrez-Lopez, L.G. (2016) Plant expression systems, a budding way to confront chikungunya and Zika in developing countries? *F1000Res*, **5**, 2121.

Chen, Q. (2016) Glycoengineering of plants yields glycoproteins with polysialylation and other defined N-glycoforms. *Proc. Natl Acad. Sci.* **113**, 9404–9406.

Chen, Q. and Davis, K. (2016) The potential of plants as a system for the development and production of human biologics. *F1000Res*, **5**, pii: F1000.

Chen, Q. and Lai, H. (2014a) Gene delivery into plant cells for recombinant protein production. *Biomed. Res. Int.* **2014**, 10.

Chen, Q. and Lai, H. (2014b) Plant-derived monoclonal antibodies as human biologics for infectious disease and cancer. In *Plant-derived Pharmaceuticals: Principles and Applications for Developing Countries*(Hefferon, K.L., ed), pp. 42–75. Croydon, UK: CABI.

Chen, Q., Lai, H., Hurtado, J., Stahnke, J., Leuzinger, K. and Dent, M. (2013) Agroinfiltration as an effective and scalable strategy of gene delivery for production of pharmaceutical proteins. *Adv. Technol. Biol. Med.* **1**, 103–112.

De Muynck, B., Navarre, C. and Boutry, M. (2010) Production of antibodies in plants: status after twenty years. *Plant Biotechnol. J.* **8**, 529–563.

Dent, M., Hurtado, J., Paul, A.M., Sun, H., Lai, H., Yang, M., Esqueda, A. *et al.* (2016) Plant-produced anti-dengue virus monoclonal antibodies exhibit reduced antibody-dependent enhancement of infection activity. *J. Gen. Virol.* **97**, 3280–3290.

Forthal, D.N., Gach, J.S., Landucci, G., Jez, J., Strasser, R., Kunert, R. and Steinkellner, H. (2010) Fc-Glycosylation Influences Fcγ Receptor Binding and Cell-Mediated Anti-HIV Activity of Monoclonal Antibody 2G12. *The Journal of Immunology.* **185**, 6876–6882.

Garber, K. (2018) No added sugar: antibody makers find an upside to 'no fucose'. *Nature Biotechnology*, **36**, 1025–1027.

Giritch, A., Marillonnet, S., Engler, C., van Eldik, G., Botterman, J., Klimyuk, V. and Gleba, Y. (2006) Rapid high-yield expression of full-size IgG antibodies in plants coinfected with noncompeting viral vectors. *Proc. Natl. Acad. Sci. USA*, **103**, 14701–14706.

He, J., Lai, H., Brock, C. and Chen, Q. (2012) A novel system for rapid and cost-effective production of detection and diagnostic reagents of west Nile virus in plants. *J. Biomed. Biotechnol.* **2012**, 1–10.

He, J., Lai, H., Engle, M., Gorlatov, S., Gruber, C., Steinkellner, H., Diamond, M.S. *et al.* (2014) Generation and analysis of novel plant-derived antibody-based therapeutic molecules against west nile virus. *PLoS ONE*, **9**, e93541.

Hiatt, A., Bohorova, N., Bohorov, O., Goodman, C., Kim, D., Pauly, M.H., Velasco, J. *et al.* (2014) Glycan variants of a respiratory syncytial virus antibody with enhanced effector function and *in vivo* efficacy. *Proc. Natl Acad. Sci.* **111**, 5992–5997.

Holtz, B.R., Berquist, B.R., Bennett, L.D., Kommineni, V.J.M., Munigunti, R.K., White, E.L., Wilkerson, D.C. *et al.* (2015) Commercial-scale biotherapeutics manufacturing facility for plant-made pharmaceuticals. *Plant Biotechnol. J.* **13**, 1180–1190.

Jefferis, R. (2005) Glycosylation of recombinant antibody therapeutics. *Biotechnol. Prog.* **21**, 11–16.

Jefferis, R. (2012) Isotype and glycoform selection for antibody therapeutics. *Arch. Biochem. Biophys.* **526**, 159–166.

Kam, Y.W., Simarmata, D., Chow, A., Her, Z., Teng, T.S., Ong, E.K., Renia, L. *et al.* (2012) Early appearance of neutralizing immunoglobulin G3 antibodies is associated with chikungunya virus clearance and long-term clinical protection. *J. Infect. Dis.* **205**, 1147–1154.

Kam, Y.-W., Pok, K.-Y., Eng, K.E., Tan, L.-K., Kaur, S., Lee, W.W.L., Leo, Y.-S. *et al.* (2015) Sero-prevalence and cross-reactivity of chikungunya virus specific anti-E2EP3 antibodies in arbovirus-infected patients. *PLoS Negl. Trop Dis.* **9**, e3445.

Lai, H. and Chen, Q. (2012) Bioprocessing of plant-derived virus-like particles of Norwalk virus capsid protein under current Good Manufacture Practice regulations. *Plant Cell Rep.* **31**, 573–584.

Lai, H., Engle, M., Fuchs, A., Keller, T., Johnson, S., Gorlatov, S., Diamond, M.S. *et al.* (2010) Monoclonal antibody produced in plants efficiently treats West Nile virus infection in mice. *Proc. Natl. Acad. Sci. USA*, **107**, 2419–2424.

Lai, H., He, J., Engle, M., Diamond, M.S. and Chen, Q. (2012) Robust production of virus-like particles and monoclonal antibodies with geminiviral replicon vectors in lettuce. *Plant Biotechnol. J.* **10**, 95–104.

Lai, H., He, J., Hurtado, J., Stahnke, J., Fuchs, A., Mehlhop, E., Gorlatov, S. *et al.* (2014) Structural and functional characterization of an anti-West Nile virus monoclonal antibody and its single-chain variant produced in glycoengineered plants. *Plant Biotechnol. J.* **12**, 1098–1107.

Lai, H., Paul, A.M., Sun, H., He, J., Yang, M., Bai, F. and Chen, Q. (2018) A plant-produced vaccine protects mice against lethal West Nile virus infection without enhancing Zika or dengue virus infectivity. *Vaccine*, **36**, 1846–1852.

Leuzinger, K., Dent, M., Hurtado, J., Stahnke, J., Lai, H., Zhou, X. and Chen, Q. (2013) Efficient agroinfiltration of plants for high-level transient expression of recombinant proteins. *J. Vis. Exp.* **77**, e50521. https://doi.org/10.3791/50521

Lomonossoff, G.P. and D'Aoust, M.-A. (2016) Plant-produced biopharmaceuticals: A case of technical developments driving clinical deployment. *Science*, **353**, 1237–1240.

Ma, J.K.C., Drossard, J., Lewis, D., Altmann, F., Boyle, J., Christou, P., Cole, T. *et al.* (2015) Regulatory approval and a first-in-human phase I clinical trial of a monoclonal antibody produced in transgenic tobacco plants. *Plant Biotechnol. J.* **13**, 1106–1120.

Moldt, B., Shibata-Koyama, M., Rakasz, E.G., Schultz, N., Kanda, Y., Dunlop, D.C., Finstad, S.L. *et al.* (2012) A Nonfucosylated Variant of the anti-HIV-1 Monoclonal antibody b12 Has enhanced FcγRIIIa-mediated antiviral activity *in vitro* but does not improve protection against mucosal SHIV challenge in macaques. *J. Virol.* **86**, 6189–6196.

Montero-Morales, L. and Steinkellner, H. (2018) Advanced plant-based glycan engineering. *Front. Bioeng. Biotechnol.* **6**, 81.

Pal, P., Dowd, K.A., Brien, J.D., Edeling, M.A., Gorlatov, S., Johnson, S., Lee, I. *et al.* (2013) Development of a highly protective combination monoclonal antibody therapy against chikungunya virus. *PLoS Pathog.* **9**, e1003312.

Prevail, II Writing Group. (2016) A randomized, controlled trial of ZMapp for ebola virus infection. *N. Engl. J. Med.* **375**, 1448–1456.

Reichert, J.M. (2012) Marketing approval of mogamulizumab AU – Beck, Alain. *mAbs*, **4**, 419–425.

Salazar-Gonzalez, J.A., Angulo, C. and Rosales-Mendoza, S. (2015) Chikungunya virus vaccines: Current strategies and prospects for developing plant-made vaccines. *Vaccine*, **33**, 3650–3658.

Schilte, C., Staikowsky, F., Couderc, T., Madec, Y., Carpentier, F., Kassab, S., Albert, M.L. *et al.* (2013) Chikungunya virus-associated long-term arthralgia: a 36-month prospective longitudinal study. *PLoS Negl. Trop Dis.* **7**, e2137.

Shevitz, J., Bonham-Carter, J., Lim, J. and Sinclair, A. (2011) An economic comparison of three cell culture techniques. *BioPharm. Int.* **24**, 1–4.

Smith, Scott A., Silva, Laurie A., Fox, Julie M., Flyak, A.I., Kose, N., Sapparapu, G., Khomandiak, S. *et al.* (2015) Isolation and characterization of broad and ultrapotent human monoclonal antibodies with therapeutic activity against chikungunya virus. *Cell. Host. Microbe.* **18**, 86–95.

Strasser, R., Altmann, F. and Steinkellner, H. (2014) Controlled glycosylation of plant-produced recombinant proteins. *Curr. Opin. Biotechnol.* **30**, 95–100.

Sun, H., Chen, Q. and Lai, H. (2018) Development of antibody therapeutics against flaviviruses. *Int. J. Mol. Sci.* **19**, 54.

Tuse, D., Tu, T. and McDonald, K. (2014) Manufacturing economics of plant-made biologics: case studies in therapeutic and industrial enzymes. *Biomed. Res. Int.* **2014**, 10.

Voss, J.E., Vaney, M.-C., Duquerroy, S., Vonrhein, C., Girard-Blanc, C., Crublet, E., Thompson, A. *et al.* (2010) Glycoprotein organization of Chikungunya virus particles revealed by X-ray crystallography. *Nature*, **468**, 709.

Weaver, S.C. and Forrester, N.L. (2015) Chikungunya: evolutionary history and recent epidemic spread. *Antiviral Res.* **120**, 32–39.

Yang, M., Dent, M., Lai, H., Sun, H. and Chen, Q. (2017a) Immunization of Zika virus envelope protein domain III induces specific and neutralizing immune responses against Zika virus. *Vaccine*, **35**, 4287–4294.

Yang, M., Lai, H., Sun, H. and Chen, Q. (2017b) Virus-like particles that display Zika virus envelope protein domain III induce potent neutralizing immune responses in mice. *Sci. Rep.* **7**, 7679.

Yang, M., Sun, H., Lai, H., Hurtado, J. and Chen, Q. (2018) Plant-produced Zika virus envelope protein elicits neutralizing immune responses that correlate with protective immunity against Zika virus in mice. *Plant Biotechnol. J.* **16**, 572–580.

Yoon, I.K., Alera, M.T., Lago, C.B., Tac-An, I.A., Villa, D., Fernandez, S., Thaisomboonsuk, B. *et al.* (2015) High rate of subclinical chikungunya virus

infection and association of neutralizing antibody with protection in a prospective cohort in the Philippines. *PLoS Negl. Trop Dis.* **9**, e0003764.

Zeitlin, L. and Haydon, H. (2015) Design and testing of ZMapp and manufacturing challenges. *Soc. Mol. Farm.* 1–37.

Zeitlin, L., Pettitt, J., Scully, C., Bohorova, N., Kim, D., Pauly, M., Hiatt, A. *et al.* (2011) Enhanced potency of a fucose-free monoclonal antibody being developed as an Ebola virus immunoprotectant. *Proc. Natl. Acad. Sci. USA*, **108**, 20690–20694.