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Characterisation of chikungunya virus neutralising monoclonal antibodies expressed in tobacco plants

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ABSTRACT

Chikungunya virus (CHIKV) causes a debilitating musculoskeletal disease, characterised by flu-like symptoms, rash, and severe joint pain, which can last for months, even after the resolution of infection. Although the first CHIKV vaccine was approved in the USA in 2023 for use in adults, there is currently no specific antiviral therapy for infection. While neutralising antibody-based prophylactic and therapeutic agents have been considered, affordability and accessibility are major barriers to global regions where Chikungunya disease is epidemic. Here, we expressed five anti-CHIKV neutralising IgG monoclonal antibodies (mAbs) in *N. benthamiana* plants to investigate the potential use of this manufacturing platform. Plants produced IgG mAbs that compared favourably to mammalian cell-expressed antibodies, including for binding kinetics to CHIKV antigens and neutralisation activity. The yields of mAbs from plants were variable, as three of the antibodies' yields would need further expression optimisation to warrant future development. The successful expression of these antibodies in *N. benthamiana* plants supports the growing pipeline of Global Health product targets that could be developed using a highly transferable, low-cost, low-tech plant production platform in resource-poor countries.

1. Introduction

Chikungunya virus (CHIKV), an alphavirus first identified in Tanzania in 1952, has spread to all six World Health Organisation (WHO)-defined regions, with new areas at risk of future outbreaks, including Europe and the USA (Rowland-Jones, 2016). While disease caused by CHIKV is not typically associated with mortality, the joint pain and musculoskeletal symptoms are debilitating in the acute phase and can progress to chronic disease lasting for months in up to 40 % of cases (Amaral et al., 2020). The first vaccine for Chikungunya virus (IXCHIQ®) was licensed only recently in November 2023 in the United States, and the European Medicines Agency in May 2024 (Maure et al., 2024). There is no specific antiviral treatment for CHIKV infection, and current approaches are focused on pain management and control of joint inflammation with use of short-term analgesics and non-steroidal anti-inflammatory drugs (Kennedy Amaral Pereira and Schoen, 2017). The mechanism and risk factors for transition to chronic Chikungunya arthritis in some patients is uncertain.

One approach that has been considered for CHIKV infection and disease is immunotherapy with neutralising monoclonal antibodies

Abbreviations: ELISA, Enzyme-Linked Immunosorbent Assay; PBS, Phosphate-buffered Saline; O.D, Optical density; ADCC, Antibody-Dependent Cellular Cytotoxicity; SPR, Surface Plasmon Resonance; MAb, Monoclonal Antibody; RU, Response Units; RLU, Relative Light Units; HRP, Horse Radish Peroxidase; SDS PAGE, Sodium Dodecyl Sulphate Poly-Acrylamide Gel Electrophoresis; IgG, Immunoglobulin G.

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(mAbs). Anti-Chikungunya mAbs have already shown promise for protection against disease in both mouse and non-human primate models (Selvarajah et al., 2013; Pal et al., 2013), and a lipid-encapsulated mRNA encoding a neutralizing anti-CHIKV human mAb has completed phase I clinical trials (August et al., 2021). Moreover, several of these mAbs were protective when administrated either 24 h before or up to 60 h post-infection even though the window of viremia is only 3–4 days. As post-exposure therapy, mAbs were effective in limiting viral spread to joints and muscles and reduced tissue inflammation in mice (Jin et al., 2015; Fox et al., 2019) and rhesus macaques (Pal et al., 2013; Broeckel et al., 2017). In humans, the acute viraemic phase for CHIKV-infection is slightly longer, lasting 4–12 days post-symptom onset, which potentially offers a longer treatment window (Appassakij et al., 2013). Chronic musculoskeletal tissue pathology is believed to be caused by persistent CHIKV infection or the presence of viral RNA. Prophylaxis with mAbs was shown to effectively prevent the establishment of CHIKV persistence (Hawman et al., 2013).

mAbs are widely used in medicine, including against cancer and chronic autoimmune disease, but are less commonly used for infectious disease applications and have almost no impact on diseases in low to middle income countries (LMICs). Palivizumab was the first commercially developed and approved antiviral mAb biologic for the prevention of respiratory syncytial virus (RSV) (Null et al., 1998) and led the way for further development of anti-RSV mAbs (Drysdale et al., 2023). Therapeutic mAbs against Ebola virus and SARS-CoV-2 have also subsequently been approved (Focosi et al., 2022; Rijal and Donnellan, 2023). Nonetheless, mAbs produced in mammalian cell cultures remain prohibitively expensive and only accessible by high income populations (Gieber et al., 2023). The issue of affordability is compounded by the need for cocktails of mAbs (*e.g.*, 3 mAbs in Inmazeb against Ebola virus) to circumvent viral escape (Rayaprolu et al., 2023), an accessibility issue highlighted by WHO in their recommendations for Ebola treatment (WHO, 2022a). While infectious diseases remain predominantly associated with LMICs, deployment of mAb-based biologicals will remain an unrealistic goal unless new approaches are established to make them affordable and accessible.

Here, we explored the feasibility of producing CHIKV neutralising mAbs in plants. Plant biotechnology offers a scalable and potentially more cost-effective approach, in which plants are used as bioreactors to produce recombinant mAbs (Moore et al., 2021, 2023). A key attraction is that a substantially lower initial investment is required to establish the manufacturing platform, compared with traditional mammalian cell culture production platforms (Stoger et al., 2014; McNulty et al., 2020). This offers LMICs the opportunity to establish their own production capacity for diseases relevant to their own region (Murad et al., 2020).

Using neutralizing mAbs that had previously been produced by mammalian cell culture, we codon-optimized mAb light and heavy chain gene sequences and expressed them in *Nicotiana benthamiana* (Teh et al., 2021). Purified proteins were characterised and compared to the same antibody produced by mammalian cells, in terms of antigen binding and breadth of viral neutralisation. Our results show that plants can be used reliably as a manufacturing platform for producing anti-CHIKV mAbs. Their use could help eventually offset the prohibitive expense of mammalian cell-derived mAb therapies, allowing the most affected regions to take ownership of treatment development programs.

2. Methods and materials

Five previously reported CHIKV neutralising IgG mAbs were studied. mAbs CHK-152, CHK-166 and CHK-263 were derived from a screen of 230 mouse mAbs and selected for their ability to inhibit infection of all three CHIKV genotypes and confer protection in a mouse challenge model (Pal et al., 2013). Two additional human mAbs against CHIKV, 5F10 and 8B10 were also selected. These mAbs were isolated from CD40-activated B cells of infected patients (Warter et al., 2011) and also protect mice from CHIKV infection (Fric et al., 2013).

2.1. Cloning

Heavy and light chain coding region sequences were obtained (Pal et al., 2013; Warter et al., 2011), and the variable region DNA sequences were plant codon-optimised and synthesised by GeneArt (Thermofisher). These heavy and light chain variable region sequences were inserted into the pTRAk.6 vector using an in-house cloning system (Pinneh et al., 2022). Briefly, heavy and light chain variable regions were digested with *NcoI/XbaI* and ligated into pWhite and pBlue entry vectors respectively, upstream of the human γ1 and κ chain constant regions already present in these vectors. This resulted in genes for mouse/human chimaeric antibodies in the case of mAbs CHK-152, CHK-166 and CHK-263. After confirmatory DNA sequencing, heavy and light chains were then both inserted, using Golden Gate cloning (*BsaI/BsmBI*), into the pTRAk.6 *Agrobacterium* binary vector. The pTRAk.6 vectors were used to transform *Agrobacterium tumefaciens* strain GV3101:pM90RK by electroporation.

2.2. N. benthamiana infiltration

Seeds from a *N. benthamiana* line that was gene edited to remove β1–2 xylosylation and α1–3 fucosylation (Jansing et al., 2019) were germinated and grown in a greenhouse with a 16/8-hour day/night cycle at 24–28°C for 4–6 weeks. Leaf infiltrations were carried out as described (Teh et al., 2013). Briefly, recombinant *Agrobacterium tumefaciens* were grown until an optical density $(O.D.)_{600\ nm}$ of 2–4 was achieved. The bacteria were pelleted and resuspended in infiltration solution (10 mM $MgCl_2$, 10 mM MES) at an $O.D._{600\ nm}$ of 0.1 and incubated at room temperature for a minimum of 30 mins with 200 μM acetosyringone. Plants were infiltrated manually using a syringe or by vacuum infiltration for larger scale expression. Plant leaves were harvested five days post infiltration for extraction of recombinant antibodies.

2.3. Gel electrophoresis

SDS-PAGE was performed following manufacturer's instructions (Invitrogen, NuPAGE), using 4–12 % Bis-Tris SDS-PAGE gels run in MOPS buffer, which were stained with InstantBlue® Coomassie Protein Stain (Abcam).

2.4. Antibody purification

Antibody was purified as described previously (Teh et al., 2013). Briefly, infiltrated plants were homogenised in a blender and filtered through miracloth (Sigma) to remove plant debris. The filtrate was centrifuged for 40 mins at 16,000 *g*, before passing the supernatant through a 0.22 μm filter. Antibodies were purified using affinity chromatography by passing the filtrates through a Protein A column (Protein A agarose, Sigma). Eluates were dialysed overnight at 4°C and concentrated by buffer exchange in 100 kDa Centricon® centrifugal filters. Antibodies were filter-sterilised and quantified using Nanodrop™ 2000 spectrophotometer (Thermofisher), before storing at 4°C, or aliquoting and storing at −80°C.

2.5. ELISAs

ELISAs were performed as previously described to confirm mAb binding activity (Webster et al., 2018). Briefly, ELISA plates were coated with 1 μg/mL of recombinant E1 or E2 CHIKV antigens (The Native Antigen Co.) and blocked with PBS + 5 % skimmed milk powder. Anti-CHIKV antibody samples (at an estimated concentration of 2 μg/mL) were added in duplicate wells with a 3-fold dilution series. After incubation, anti-CHIKV antibodies were detected with anti-human IgG1 heavy chain antiserum conjugated with HRP (The Binding Site, cat. #AP015), diluted in PBS + 5 % skimmed milk powder. Developing

solution (3,3 5,5-Tetramethylbenzidine (TMB) Liquid Substrate, Sigma) was added and briefly incubated until colour development was complete before stopping with 2 N H_2SO_4. Plates were read on a Tecan Infinite F200 Pro. Data were analysed and concentrations calculated with Graphpad Prism 9. A minimum of three biological repeats was performed for each assay.

2.6. Binding kinetics

Surface plasmon resonance (SPR) was used to calculate binding kinetics of the antibodies to CHIKV antigens, using a BIAcore™ X-100 instrument (GE healthcare, Chalfont St. Giles, UK) (Stelter et al., 2020). All proteins were diluted/resuspended in HBS-EP+ buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.05 % surfactant P-20) at 25°C. Protein A (Sigma, cat. #P6031) was immobilised onto a CM5 chip with standard amine coupling, to 5000 response units. Antibodies CHK-152 and CHK-263 were individually captured onto a protein A-coated sensor chip and recombinant CHIKV-E2 protein was injected over the antibody surface at a range of concentrations (0.2–0.8 μM). Sensorgrams were analysed with a 1:1 binding model to fit the binding behaviour, followed by 1 h of dissociation time and a regeneration step with 10 mM glycine-HCl (pH 1.5). All referenced and blanked sensograms were fitted to Langmuir model of binding (1:1), using BIAcore™ Evaluation software.

For CD16 and CD64 binding kinetics, CHIKV mAbs were captured by Protein A on CM5 chips (as above). Fc receptors (Recombinant Human Fc gamma RIIIA/CD16a Protein and Recombinant Human Fc gamma RI/CD64 Protein, Bio-techne) were injected over the antibody surface at a range of concentrations (0.2–0.8 μM). Kinetic parameters were determined using a 1:1 binding model to fit the binding behaviour.

2.7. Neutralisation assays

Serial dilutions of antibodies were incubated with 100 focus forming units (FFU) of CHIKV (strain LR2006_OPY1) for 1 h at 37°C then added to Vero cell monolayers for 1.5 h at 37°C. The cells were then overlayed with 1 % methylcellulose in minimum essential medium supplemented with 2 % heat inactivated FBS, HEPES, and penicillin and streptomycin. After 18 h, the cells were fixed with 1 % paraformaldehyde in PBS and then permeabilized. The cells were then stained with CHK-11 mAb (Pal et al., 2013) overnight followed by an anti-mouse IgG conjugated to peroxidase (SeraCare) for 2 h at room temperature. Foci were developed with TrueBlue Peroxidase Substrate (KPL) and counted on a Biospot plate reader (Cellular Technology, Inc.). IC_{50} values were determined by non-linear regression with the top and bottom constrained to 100 and 0, respectively. The focus reduction neutralization tests were performed under BSL-3 conditions.

3. Results

3.1. mAb expression

The plant codon-optimised genes for the five anti-CHIKV mAbs resulted in different expression levels. Pre-purification yields were 64.7 mg/kg (fresh leaf mass) for the chimaeric CHK-152, 49.6 mg/kg for chimaeric CHK-263, 27 mg/kg for chimaeric CHK-166 antibodies, and 35 mg/kg and 15 mg/kg for the human 8B10 and 5F10 mAbs respectively.

Fig. 1 shows a Coomassie stained SDS-PAGE gel of the anti-CHIKV mAbs expressed and purified from *N. benthamiana*. The positive control is a commercial purified human IgG1κ mAb. Major bands around 150 kDa were detected under non-reducing (N) conditions, the expected size for fully assembled IgG mAbs. Some evidence for degradation was observed for CHK-152, CHK-263, and 8B10. Under reducing (R) conditions, individual heavy and light chains are detected at the expected sizes of approximately 55 kDa and 27 kDa.

3.2. Antigen binding

All five mAbs demonstrated specific binding to CHIKV antigens (Fig. 2**A**). There was no binding to LDH, a non-specific antigen (data not shown). CHK-166 recognised recombinant E1 protein, whereas CHK-152, CHK-263, 5F10 and 8B10 showed binding to recombinant E2 protein.

Detection of antigen binding by CHK-152 and CHK-263 appeared less potent than for 5F10 and 8B10, so the binding kinetics for E2

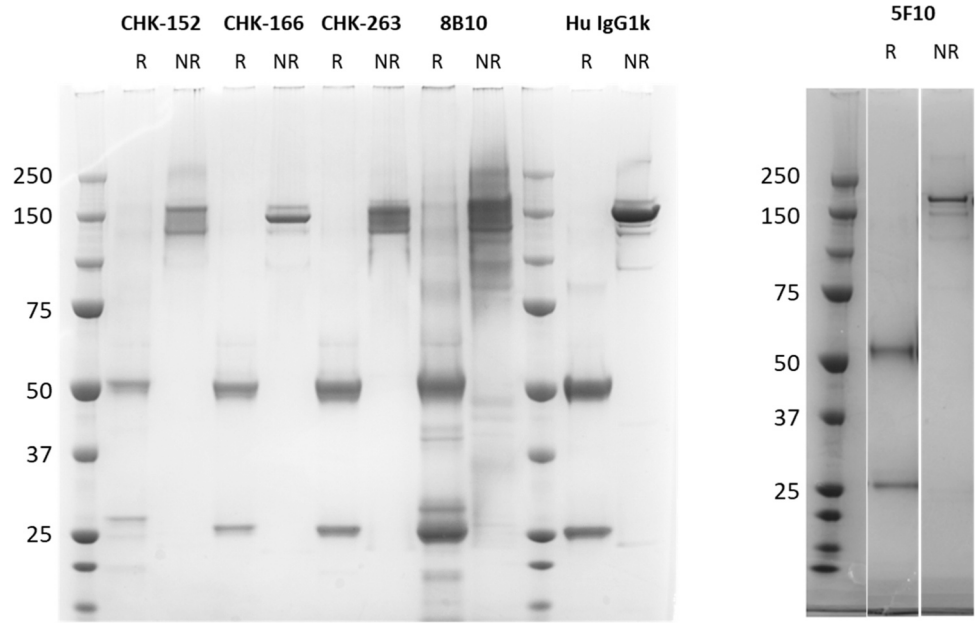


Fig. 1. Purified anti-CHIKV antibodies produced in *N. benthamiana*. Purified plant-produced CHK-152, CHK-166, CHK-263, 5F10, 8B10 mAbs and purified human IgG1 kappa (Sigma) were electrophoresed under non-reducing (NR) and reducing (R) conditions on 4–12 % SDS-PAGE gels. Molecular weight standards (Precision Plus Protein™ All Blue Pre-stained Protein Standards) are indicated. (The marker and two lanes for 5F10 were from the same gel, but intervening samples have been removed for the sake of clarity.). Representative gel images are shown from 3 replicate experiments.

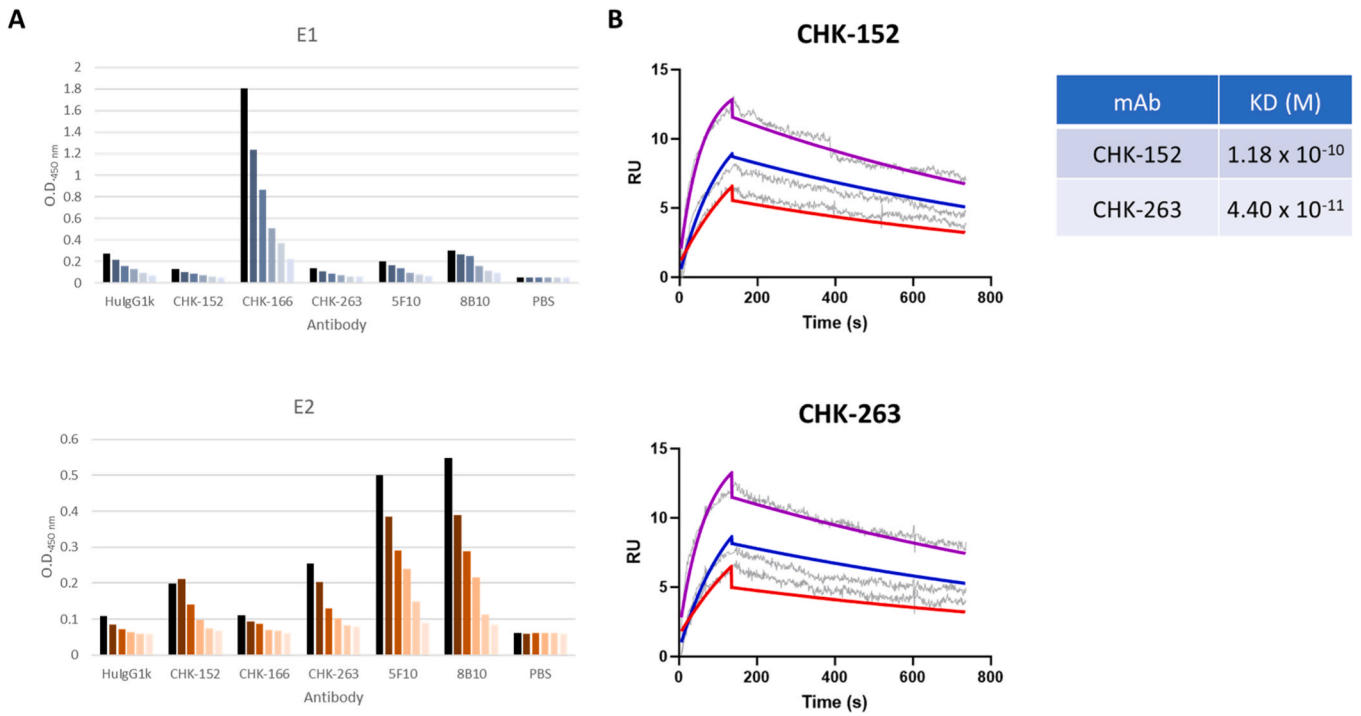


Fig. 2. Antigen specific binding of purified anti-CHIKV antibodies. (A) Antigen binding ELISAs. Recombinant antigens E1 and E2 were used to coat the ELISA plates. Anti-CHIKV mAbs were added in duplicate wells at a starting concentration of ~220 ng/mL for E1 ELISAs and ~70 ng/mL for E2 ELISAs, with a 3-fold dilution series. Bound antibodies were detected using HRP-conjugated goat anti-human IgG Fc domain antibody. Data shown as absorbance for mAbs at 450 nm (mean of replicate samples). (B) Surface plasmon resonance for CHK-152 and CHK-263 mAbs binding to recombinant CHIKV E2. Concentrations were 0.8 μM (Purple), 0.4 μM (Blue), and 0.2 μM (red). Association (ka) and dissociation (kd) constants were calculated using the Langmuir 1:1 binding model with BIAcore evaluation software, K_D = ka/kd. Data shown is representative of 3 independent experiments.

antigen were explored in more detail by surface plasmon resonance (Fig. 2B). Binding to E2 was confirmed and the affinities (K_D) for the plant-expressed CHK-152 and CHK-263 antibodies were calculated as 1.18×10^{-10} M, and 4.40×10^{-11} M respectively.

3.3. Binding to Fcγ receptors

The binding kinetics to the high affinity FcγRI (CD64) receptor and a low affinity FcγRIIIa (CD16) receptor were measured for all five mAbs by surface plasmon resonance (Fig. 3). The results were consistent for all antibodies, with a K_D range of $4.63–5.31 \times 10^{-10}$ for CD64 and $2.64–3.45 \times 10^{-8}$ for CD16, with the latter results being consistent for non-fucosylated IgG mAbs (Webster et al., 2018).

3.4. Neutralisation assays

To measure neutralisation of Chikungunya virus, we performed focus reduction neutralization tests (FRNT) (Fig. 4). For mAbs CHK-152, CHK-166 and CHK-263, a direct comparison between antibodies produced in plants or from hybridomas was made and the FRNT-derived IC_{50} values were comparable (Fig. 4A). The IC_{50} values for plant-produced 8B10 and 5F10 were 32 and 36 ng/mL respectively (Fig. 4B), consistent with published values of these antibodies (Warter et al., 2011).

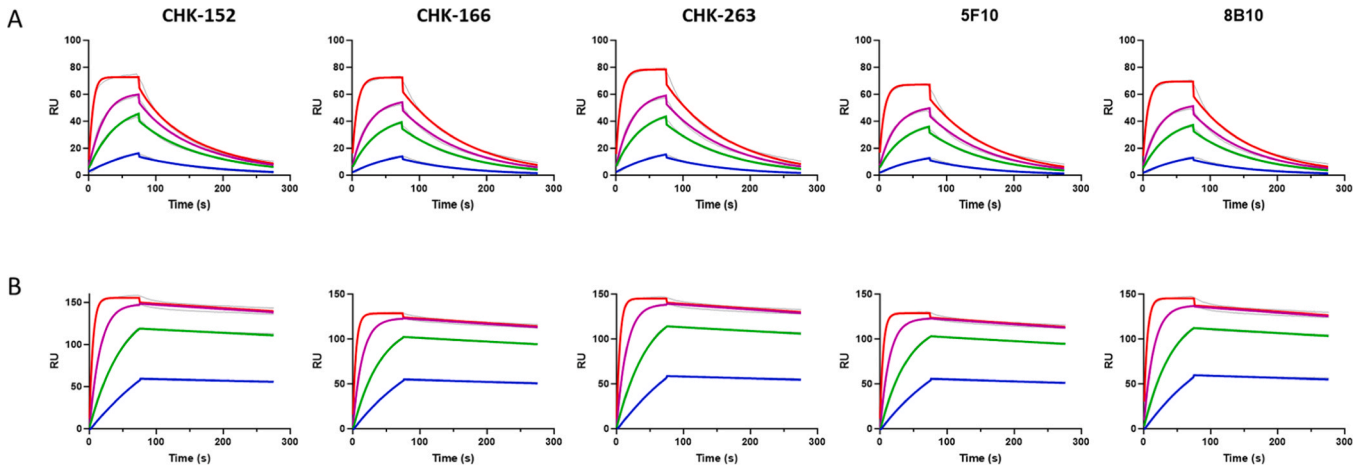
4. Discussion

Since its initial discovery more than 70 years ago, CHIKV has periodically caused epidemics in Africa and Asia. In 2005–2006, a major outbreak in the Indian ocean region later led to imported cases across Asia, Australia, North America and Europe. In 2007, a local outbreak of Chikungunya disease appeared for the first time in Southern Europe, and there have since been further local outbreaks in France. In 2013, CHIKV spread to the Caribbean and South America, and the virus currently circulates across the sub-tropical regions of America, Africa and Asia (WHO, 2022b). CHIKV is primarily transmitted to people by infected mosquitoes, and the risk of global spread is increasing due to importation through infected travellers, presence of competent vectors in many countries (particularly around the Mediterranean coast), global warming, and population susceptibility (Watson, 2007). Thus, CHIKV is an emerging global health threat. In 2024, as of 30th September, approximately 460,000 chikungunya virus disease cases and 170 CHIKV associated deaths were reported worldwide from 23 countries (ECDC https://www.ecdc.europa.eu/en/chikungunya-monthly).

Current options to manage Chikungunya infection outbreaks are limited. The first anti-CHIKV vaccine (IXCHIQ®) was approved in November 2023, but it is currently only licensed for use in adults in Europe and the USA. It is a live-attenuated vaccine, and severe chikungunya-like adverse reactions were observed in some recipients. As a consequence, a post-marketing study was requested to assess this serious potential risk (Ly, 2024).

Experience with other vaccines for tropical diseases suggests that affordability will be a key consideration as to whether the vaccine is widely used in the low- and middle-income country settings where it is most needed (Guillaume et al., 2023). IXCHIQ® also does not provide a protection strategy for children. In addition, vaccine hesitancy is a growing concern worldwide, so further clinical options are urgently needed (Nuwarda et al., 2022). There is no specific antiviral drug treatment for CHIKV infection.

Whilst mammalian cell-derived mAbs could be used in multiple ways to manage Chikungunya infection, they are expensive and currently even less accessible to LMICs than vaccines (Eaton et al., 2016). An additional complicating factor is that a single mAb may not provide sufficient protection against all circulating viral strains, and viral escape may also develop. For these reasons, mAb combinations or cocktails are generally considered advisable (Pal et al., 2013), but this potentially multiplies the cost of a drug product.



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mAb	CD16 KD (M)	CD64 KD (M)
CHK-152	2.64×10^{-8}	4.63×10^{-10}
CHK-166	3.39×10^{-8}	5.29×10^{-10}
CHK-263	3.28×10^{-8}	4.76×10^{-10}
5F10	3.43×10^{-8}	5.31×10^{-10}
8B10	3.45×10^{-8}	5.28×10^{-10}

Fig. 3. Surface plasmon resonance measuring binding kinetics of anti-CHIKV mAbs to CD16 and CD64. Protein A-coated CM5 surface was used to bind CHIKV mAb, and the soluble Fc receptor was added in the fluid phase. (A) Binding to CD16a (FcγRIIIa). Concentrations added were 400 nM (red), 100 nM (purple), 50 nM (green), and 12.5 nM (blue). (B) Binding to CD64 (FcγRI). Concentrations added were 270 nM (red), 90 nM (purple), 30 nM (green), and 10 nM (blue). (C) Kinetic parameters from 1:1 Langmuir curve fitting. Affinities (K_D) from surface plasmon resonance were estimated using the Langmuir model of binding (1:1), with BIAcore™ Evaluation software. The results are representative of three independent experiments.

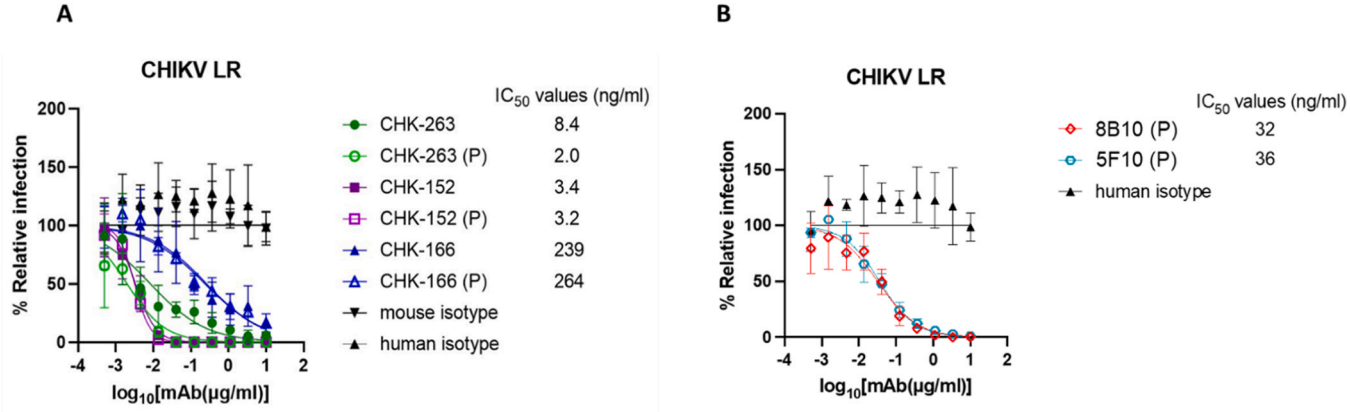

Fig. 4. Neutralization of CHIKV by mAbs. Focus reduction neutralization tests (FRNT) were performed by incubating indicated mAbs with 10^2 focus-forming units of CHIKV (strain LR2006_OPY1) before adding to Vero cells. The cells were permeabilised and stained with mAb CHK-11. Foci were counted on a Biospot plate reader. IC_{50} values are shown. (A) mAbs CHK-152, CHK-166 and CHK-263 produced in plants (P) were compared with those produced in hybridomas. A human and mouse isotype IgG was included as a negative control. (B) mAbs 8B10 and 5F10 were produced in plants (P). A human isotype IgG was included as a negative control. Graphs show the mean ± SD for two independent experiments performed in duplicate. IC_{50} values were determined with non-linear regression constraining the top and bottom to 100 and 0, respectively.

Plant manufacturing platforms for protein biologics are emerging as an alternative to conventional cell culture systems, particularly as manufacturing facilities are potentially much more affordable for resource poor regions (Murad et al., 2020). Manufacturing in plants has been used for the production of Ebola mAbs (Group et al., 2016) and a COVID vaccine (Hager et al., 2022) in North America, providing proof-of-concept and demonstrating a potential regulatory path forward. As a consequence, the recent establishment of plant manufacturing facilities for biologics in resource poor regions, such as Baiya Phytopharma in Thailand (baiyaphytopharm.com), Cape Biologix in South Africa (capebiologix.com) and the Bio-Manguinhos/Fiocruz Centre in Ceara (ceara.fiocruz.br/portal/index.php/inovacao-e-producao/bio-manguinhos/) heralds the beginning of a potentially important shift in biologics manufacture, that could allow LMICs to determine and

prioritise their own healthcare needs.

Several promising Chikungunya neutralising mAbs have been reported (Chandley et al., 2023), including in plants. Hurtado et al., previously reported the expression of a CHIKV neutralising mAb in plants and demonstrated *in vitro* and *in vivo* efficacy (Hurtado et al., 2020). Esqueda et al., also reported a bispecific antibody against dengue and chikungunya virus in plants, using the CHK-152 mAb sequence (Esqueda et al., 2021). In our study, anticipating the possible need for a mAb cocktail drug product, we expressed five different anti-CHIKV neutralising mAbs by transient expression in *N. benthamiana*, and in three cases demonstrated a functional equivalence of virus neutralisation with the same antibody sequences purified from hybridoma supernatant.

The antibodies selected here were based on their epitope specificities, potent neutralisation activity, and protective activity in animal models. Our data confirm that the antigen specificity of the plant-produced version of these mAbs was preserved. mAbs 8B10 and 5F10 bind distinct epitopes on E2 (Porta et al., 2016). mAb 8B10 binds radially from the viral surface and blocks receptor binding on the E2 glycoprotein. mAb 5F10 binds the tip of the E2 B domain and fixes the B domain rigidly to the surface of the virus, blocking exposure of the fusion loop on glycoprotein E1 and therefore preventing the virus from becoming fusogenic (Porta et al., 2016). mAbs CHK-152 and CHK-263 were mapped to distinct epitopes on the E2 protein, and CHK-166 binds an epitope on the E1 protein (Pal et al., 2013). Analysis of amino acid changes in escape mutants showed that neutralization escape residues for CHK-263 localised to the B domain of E2 (Pal et al., 2013). The residues that modulated CHK-152 neutralization mapped to the A domain of E2. In contrast, CHK-166 recognized amino acids on DII of E1, adjacent to the fusion loop. Furthermore, low resolution cryo-electron microscopy structures of CHIKV VLPs in complex with Fab fragments revealed that CHK-152 spans E2 domain A and domain B as well as the β-ribbon connector connecting the two domains within one E2 molecule (Sun et al., 2013).

In vivo, mAbs 8B10 and 5F10 were previously shown to protect mice from CHIKV infection (Fric et al., 2013). mAbs CHK-152, CHK-166 and CHK-263 were also protective in a pre-exposure mouse model. Furthermore, in a post-exposure therapeutic mouse model, administration of CHK-263 +CHK-152 or CHK-166 +CHK-152 at 24 h post infection completely prevented mortality and emergence of resistance in all animals (Pal et al., 2013). The combination mAb therapy of CHK-152 and CHK-166 has also been tested in a nonhuman primate model of CHIKV infection. Rhesus macaques treated with these antibodies had reduced viral spread and infection, with neutralized reservoirs of infectious virus. No escape viruses were detected (Pal et al., 2014). The results from animal studies are therefore supportive that selected pairs of highly neutralizing MAbs may be a promising treatment option for CHIKV in humans.

We found that the each anti-CHIKV mAb was expressed with different yields in plants. This is not unexpected, even though the only difference in sequence lies in the variable regions of the antibodies (Niemer et al., 2014). The best expressing antibodies in plants were CHK-152 and CHK-263, which fortuitously have the strongest neutralizing activities. These two mAbs are clear candidates for development, whereas further yield optimisation is likely needed before the other three mAbs can be advanced, as their initial yields were below the lower limit for manufacturing feasibility (Moore et al., 2021). With regards to downstream purification, we used Protein A affinity chromatography successfully, which is a key step in mAb manufacture. Previous work has identified a path for further upstream and downstream processing, to reach the product quality required for use in human clinical trials (Sparrow et al., 2007; Ma et al., 2015).

Functionally, the plant produced mAbs performed as expected. Antigen binding was confirmed by ELISA and in the case of mAbs CHK-152 and CHK-263 binding kinetics derived from SPR experiments confirmed high binding affinity in the sub-nanomolar range (Pal et al., 2013).

N-linked glycosylation in plants is well understood and has been the subject of much investigation (Cabanes-Macheteau et al., 1999). IgG mAbs produced in plants have been reported to have much better homogeneity on N-linked glycans (Ma et al., 2015). Furthermore, the glycoengineering strategy used in this study, which is commonly used for plant expression, eliminates N-linked glycan residues (β1,2-linked xylose, core α1,3-linked fucose) that are commonly found in plants, but not humans. Thus, the resulting IgG mAb only displays N-linked glycans that are also found in normal human IgG (Jansing et al., 2019). De-fucosylation of IgG antibodies leads to an improvement in binding affinity to the low affinity CD16a Fc receptor, that is found on natural killer cells and linked with ADCC (Coenon and Villalba, 2022). We confirmed the binding affinity of the plant CHIKV mAbs to high and low affinity Fcγ receptors CD64 and CD16, which was consistent with previously published results (Stelter et al., 2020). This is pertinent because previously it was shown that the effector functions of at least CHK-152 contribute to protection *in vivo* (Pal et al., 2013). When CHK-152 and CHK-166 were administered as post-exposure therapy in mice, the Fc effector functions of both mAbs promoted virus clearance from infected cells and reduced joint swelling (Fox et al., 2019).

Finally, virus neutralisation activity was tested. CHK-152, CHK-166 and CHK-263 plant antibodies all performed equivalently compared to their mammalian cell-produced counterparts. For plant-produced 8B10 and 5F10, IC50 values were consistent with previously reported historical data from CHO derived mAbs (Pal et al., 2013; Warter et al., 2011).

Overall, this study demonstrates that murine/human chimeric and human anti-CHIKV antibodies can be efficiently expressed in tobacco plants without loss of function. This raises the prospect of developing a low-cost, low-tech production platform for a mAb cocktail to control chikungunya disease in low- to middle-income countries.

CRediT authorship contribution statement

Catherine M. Moore: Writing – review & editing, Writing – original draft, Supervision, Investigation, Formal analysis, Data curation. **Julian K-C. Ma:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Julie M. Fox:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Michael S. Diamond:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Roger Hewson:** Investigation. **Sathishkumar Ramalingam:** Formal analysis. **James Ashall:** Investigation, Data curation. **Balamurugan Shanmugaraj:** Investigation, Formal analysis, Data curation. **Elizabeth Pinneh:** Investigation, Formal analysis, Data curation. **Mathew J. Paul:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation.

Ethical approval

No ethical approval was required.

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Sequence information

N/A

Declaration of Competing Interest

M.S.D. is a consultant or advisor for Inbios, Vir Biotechnology, IntegerBio, Moderna, Merck, and GlaxoSmithKline. The Diamond laboratory has received unrelated funding support in sponsored research agreements from Vir Biotechnology, Emergent BioSolutions, and IntegerBioAll co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report.

Data availability

Data will be made available on request.

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