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Chikungunya Virus Envelope-Specific Human Monoclonal Antibodies with Broad Neutralization Potency

Lucile Warter,* Chia Yin Lee,* Rekha Thiagarajan,* Marc Grandadam,† Serge Lebecque,‡ Raymond T. P. Lin,§ Sebastien Bertin-Maghit,⁎ Lisa F. P. Ng,* Jean-Pierre Abastado,⁎ Philippe Desprès,† Cheng-I Wang,⁎† and Alessandra Nardin*,1

Chikungunya virus (CHIKV) is an alphavirus responsible for numerous epidemics in Africa and Asia. Infection by CHIKV is often characterized by long-lasting, incapacitating arthritis, and some fatal cases have been described among elderly and newborns. Currently, there is no available vaccine or specific treatment against CHIKV. Blood B cells from a donor with history of CHIKV infection were activated, immortalized, amplified, and cloned. Two human mAbs against CHIKV, 5F10 and 8B10, were identified, sequenced, and expressed in recombinant form for characterization. In a plaque reduction neutralization test, 5F10 and 8B10 show mean IC₅₀ of 72 and 46 ng/ml, respectively. Moreover, both mAbs lead to a strong decrease in extracellular spreading of infectious viral particles from infected to uninfected cells. Importantly, the mAbs neutralize different CHIKV isolates from Singapore, Africa, and Indonesia, as well as O’nyong-nyong virus, but do not recognize other alphaviruses tested. Both mAbs are specific for the CHIKV envelope: 5F10 binds to the E2 glycoprotein ectodomain and 8B10 to E1 and/or E2. In conclusion, these two unique human mAbs strongly, and specifically neutralize CHIKV infection in vitro and might become possible therapeutic tools against CHIKV infection, especially in individuals at risk for severe disease. Importantly, these mAbs will also represent precious tools for future studies on host–pathogen interactions and the rational design of vaccines against CHIKV. The Journal of Immunology, 2011, 186: 000–000.
To develop a CHIKV-specific treatment based on passive immunization strategies, we endeavored to isolate and characterize CHIKV-neutralizing human mAbs. CD40-activated peripheral blood B cells from an individual with prior CHIKV infection were immortalized by EBV infection for the subsequent identification and cloning of CHIKV-neutralizing B cells. Two CHIKV-neutralizing mAbs were isolated, expressed as recombinant IgG1, and further characterized. The mAbs described in this study, both specific for the CHIKV envelope, demonstrated high in vitro neutralization potency, in both preventive and postinfection settings, broad activity against several CHIKV isolates, and lack of cross-reactivity with other alphavirus except with O’nyong-nyong virus (ONNV).

Materials and Methods

Cell culture

B cells were grown in DMEM-F12 with 10% FCS, l-glutamine, penicillin, and streptomycin (all from Life Technologies-Invivogen) and 2% ADCC (Dendritics). HEK293T (ATCC No. CRL-N268) and Vero cells (ATTC No. CCL-81) were grown in DMEM with 10% FCS (DMEM). The HEK293TPM1 cells (gift from Dr. John Wu, Promab Biotechnologies), a HEK293T cell subclone selected to be highly transfectable, were grown in F17-medium with l-glutamine, Geneticin, and Pluronic F68 (all from Life Technologies-Invivogen).

Plasmids, Abs, and recombinant proteins

The expression plasmid pPMhIgG1 (gift from Dr. John Wu, Promab Biotechnologies) encodes the mouse IgG signal peptide and the human IgG1 C region (CHI-3), cloned between Nhel and NotI restriction sites. A Sall restriction site is located downstream of the peptide sequence.

The mouse mAb 3E4 is specific for CHIK06-49 E2 protein (28). The human IgG1 HA4 (kindly provided by DSO National Laboratories, Singapore) is specific for H5N1 influenza virus.

The recombinant CHIK E2 was previously described (28). In this study, we used 

\[ \text{e2-SNAP} ]

consisting of CHIK e2 fused to SNAP (Covalys BioSciences AG) followed by a hexa-histidin tag, and expressed in Drosophila S2 cells. The soluble 

\[ \text{e2-SNAP} ]

secreted into the extracellular medium of induced S2 cells for 10 d, was purified on chelating column chromatography and then Superdex column. The recombinant E1 protein contains the ectodomain of CHIKV E1 glycoprotein fused upstream of hexa-histidin tag. The E1-hexa-histidin tag encoding sequence was molecularly cloned into pPMhIgG1, between Sall and NotI restriction sites, for expression in HEK293TPM1 cells.

Viruses

The two CHIKV isolates CHIK/Singapore/07/2008 and CHIK/Singapore/11/2008, referred as CHIKV07 and CHIKV11, respectively, were isolated from patients at the National University of Singapore. CHIKV strains were amplified in Vero cells. Viral stocks were titrated using a standard plaque assay.

All alphaviruses except the Singapore CHIKV isolates and the Indonesia CHIKV isolate (Institut de Médecine Tropicale du Service de Santé des Armées, Marseille, France) were provided by the National Reference Center for Arboviruses, Institut Pasteur (Paris, France).

The EBV stock was produced from supernatant of B95.8 cells (ATCC No. VR-1492).

Isolation of CHIKV-neutralizing B cell clones

The research was approved by the Institutional Review Board. Forty-five milliliters of blood were obtained with informed consent from one individual previously infected by CHIKV and who subsequently fully recovered. PBMCs were isolated by Ficoll, and the percentage of CD19+ cells expressing CD40 ligand (Dendritics). The activated B cells were then immortalized by EBV infection by adding 100 μl/well B95.8 cells supernatant. Two weeks later, supernatants from the polyclonal B cell populations were analyzed by binding and neutralization immunofluorescent assays. Immortalized CHIKV-neutralizing B cells were cloned by limiting dilution.

mAb purification

Monoclonal B cells were cultured for 7 d in complete DMEM-F12 medium without FCS. Culture supernatants were incubated overnight with protein G agarose (Millipore). The bound Abs were eluted with glycine solution and dialyzed in PBS. Ab concentration was determined by spectrophotometry (Nanodrop). The Ab isotype was determined by flow cytometry using the Multiplex Bead Assay for Human Isotyping (Southern Biotech). The purification of recombinant mAbs from the supernatant of HEK293TPM1 cells was similar.

mAb sequencing

Total RNA of B cell cultures was extracted using TRIzol reagent (Invitrogen). From each RNA preparation, two independent reverse transcriptions were performed using SMARTer CDNA synthesis Kit (Clontech). Each cDNA was PCR-amplified using Advantage 2PCR Kit (Clontech) and a combination of primers specific for all the heavy and light chains. Amplified heavy and light chain PCR products were independently cloned into TOPO vector (Invitrogen). DNA was purified from 15 bacterial colonies and sequenced. A consensus sequence was determined by alignment of the 15 independent sequences.

Recombinant mAb expression

The consensus nucleotide sequences encoding the variable domain of the heavy chains and the whole light chains were molecularly cloned into pPMhIgG1 plasmid, between Nhel-Sall and Nhel-NotI restriction sites, respectively. The plasmids encoding the heavy and light chains were cotransfected into HEK293TPM1 cells using lipofectamine 293 (Invitrogen).

Binding and neutralization immunofluorescent assays

Binding test. HEK293T cells, previously seeded into 96-well plates, were infected with CHIKV or other alphaviruses at a multiplicity of infection of 0.1. The day after, cells were washed and fixed with a 7/3 ethanol/acetone solution. B cell culture supernatants or mAbs were added to CHIKV-infected and, as a negative control, to uninfected cells for 1 h at 37°C. Anti-CHIKV polyclonal plasma (1:200) and human serum AB (Gemini-Bioproducts, 1:200) or 1 μg/ml HA4 mAbs were added as positive and negative control, respectively. The binding of anti-CHIKV Abs was determined with a mixture of Alexa 488-labeled anti-human IgG, IgM, IgA Abs and with Alexa488 anti-human IgG (all from Invitrogen) for B cell supernatants and purified/recombinant mAbs, respectively.

Neutralization test. Four thousand CHIKV11 PFUs were incubated for 1 h at 37°C with equal volume of CHIKV-specific B cell supernatants or 2 μg/ml mAbs. Anti-CHIKV human plasma (1:10) and human serum AB (1:10) or 2 μg/ml HA4 mAbs were used as positive and negative control, respectively. The mixtures were added onto 40,000 HEK293T cells for 1.5 h. Twenty-four hours later, the cells were fixed, blocked, and incubated with anti-CHIKV plasma (1:200), followed by anti-human Ab mixture.

Fluorescence was analyzed under a fluorescent microscope (Nikon ECLIPSE TS 100) at 100 magnification.

Plaque reduction neutralization test and determination of mAb potency

One hundred to 200 CHIKV PFUs were mixed with different concentrations of mAbs. After 1 h at 37°C, the mixtures were added onto Vero cells for 1.5 h, then replaced by DMEM-0.25% agarose for 2 d. PFUs were counted after crystal violet staining. The percent neutralization was calculated as follows:

\[
\text{Percent neutralization} = \left(1 - \frac{PFU_{\text{mAb of interest(all concentrations)}}}{PFU_{\text{negative control(all concentrations)}}}\right) \times 100
\]

Neutralization curves were generated and analyzed using GraphPad Prism 5. Nonlinear regression fitting with sigmoidal dose–response (variable slope) was used to determine the IC50 and IC90. Nonparametric Friedman’s test was used to compare the potency of mAbs and their combination.

Postinfection neutralization assay

HEK293T cells were infected with CHIKV11 (multiplicity of infection = 0.1). Eleven hours postinfection, cells were extensively washed and different concentrations of mAbs were added. After 2 h, cells were washed and
Recombinant 5F10 and 8B10 mAbs have similar CHIKV-neutralizing activity compared with their B cell-purified counterparts, the recombinant mAbs were used in further characterization studies.

The in vitro potency of the recombinant mAbs and their combination was evaluated by plaque reduction neutralization test over a range of concentrations, from 100 pg/ml to 200 μg/ml. Fig. 2B shows the neutralization curves calculated using data from five independent experiments; IC_{50} and IC_{80} (the concentration required to obtain 50% or 80% of the maximum measured effect, respectively) from each individual experiment are presented in Fig. 2B, and means and SD in Table I. There were no significant differences in the potency of the two mAbs or their combination: IC_{50} ranged between 10 and 200 ng/ml, and IC_{80} between 70 ng/ml and 1.7 μg/ml.

Thus, 5F10 and 8B10 are equally potent against the CHIKV11 isolate, and their effect is neither synergistic nor additive, suggesting that their mechanism of neutralization and/or recognized epitope might be similar. 5F10 and 8B10 mAbs inhibit the extracellular spreading of infectious viral particles from CHIKV-infected cells

With the purpose of investigating the in vitro potency of 5F10 and 8B10 mAbs in an assay potentially more relevant for a treatment setting, we next assessed whether the mAbs are also capable of preventing viral spreading from infected to uninfected cells.

HEK293T cells infected with CHIKV11, then treated for 2 h with different concentrations of mAbs. The amount of infectious CHIKV11 particles present in the media was determined 2 and 6 h after treatment.

**Results**

**Isolation of human mAbs neutralizing CHIKV infection in vitro**

PBMCs were collected from a donor 6 wk after CHIKV infection. B cells were activated by CD40 ligation, immortalized by EBV infection, and expanded. B cell lines whose supernatant was neutralizing CHIKV in the immunofluorescence assay were cloned by limiting dilution, rescreened, and expanded for production of anti-CHIKV Abs. The monoclonality of two positive clones, 5F10 and 8B10, was confirmed by nucleotide sequencing; their isotype was IgG1k2 and IgG1k1, respectively. Both mAbs were able to bind to CHIKV11 and block infection at concentrations of 1 μg/ml (Fig. 1A, 1B).

Recombinant IgG1 mAbs were next produced, and their neutralization capacity was compared with that of the corresponding B cell-derived mAbs. The neutralizing potency of recombinant and purified mAbs against the CHIKV11 isolate was similar, as assessed by a quantitative plaque reduction neutralization test (Fig. 1C).

**In vitro potency of recombinant anti-CHIKV mAbs**

Having demonstrated that the recombinant 5F10 and 8B10 mAbs have similar CHIKV-neutralizing activity compared with their B cell-purified counterparts, the recombinant mAbs were used in further characterization studies.

The in vitro potency of the recombinant mAbs and their combination was evaluated by plaque reduction neutralization test over a range of concentrations, from 100 pg/ml to 200 μg/ml. Fig. 2A shows the neutralization curves calculated using data from five independent experiments; IC_{50} and IC_{80} (the concentration required to obtain 50% or 80% of the maximum measured effect, respectively) from each individual experiment are presented in Fig. 2B, and means and SD in Table I. There were no significant differences in the potency of the two mAbs or their combination: IC_{50} ranged between 10 and 200 ng/ml, and IC_{80} between 70 ng/ml and 1.7 μg/ml.

Thus, 5F10 and 8B10 are equally potent against the CHIKV11 isolate, and their effect is neither synergistic nor additive, suggesting that their mechanism of neutralization and/or recognized epitope might be similar. 5F10 and 8B10 mAbs inhibit the extracellular spreading of infectious viral particles from CHIKV-infected cells

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HEK293T cells infected with CHIKV11, then treated for 2 h with different concentrations of mAbs. The amount of infectious CHIKV11 particles present in the media was determined 2 and 6 h after treatment.
post-treatment. After 2 h, 1 μg/ml 5F10, 8B10, or a combination led to a PFU decrease of 56 to 87% (Fig. 3). After 6 h, the antiviral effect of 5F10 and/or 8B10 mAbs was lower, as mAb concentrations ranging from 100 ng/ml to 100 μg/ml were required to induce a PFU decrease of 43 to 72% (Fig. 3). As previously seen by plaque reduction neutralization test (Fig. 2), the mAb combination does not induce stronger protection compared with the single mAbs.

These results indicate that both mAbs are capable of inhibiting extracellular spreading of infectious viral particles from infected to uninfected cells. The mAb binding might inhibit virus budding at the plasma membrane. Alternatively, the loss of infectivity might be caused by the Ab-mediated capture of newly released virus particles from the host cells. Therefore, 5F10 and 8B10 mAbs might be usable not only to prevent CHIKV infection, but also to limit CHIKV extracellular spreading and possibly disease severity.

Antigenic specificity of 5F10 and 8B10

The specificity of 5F10 and 8B10 mAbs was studied by Western blot. The CHIKV-specific mAbs and the anti-CHIKV plasma were treated for 2 h with recombinant mAbs as 5F10 and 8B10 mAbs are capable of immunoprecipitating both E2 and its precursor pre-E2 (molecular mass 62 kDa), as revealed by the E2-specific murine mAb 3E4. We next investigated in Western blot the recognition of the recombinant CHIKV E1 protein by the mAbs. The anti-CHIKV plasma, but not 5F10 or 8B10, recognized a band corresponding to E1 (Fig. 4D). The results shown in Fig. 4C and 4D suggest that 8B10, like 5F10, binds to E2. However, we cannot rule out a possible communoprecipitation of the E1/E2 heterodimer without any direct 8B10–E2 interaction (Fig. 4C) or potential conformational differences between the soluble E1 and the native CHIKV E1 proteins, which might prevent, under our experiment conditions, the binding of E1 by 8B10 (Fig. 4D).

Overall, our results demonstrate that 5F10 binds to E2, whereas 8B10 binds to E1 and/or E2, but more likely E2. Moreover, despite the lack of synergy observed in the neutralization assays, the epitopes recognized by the two mAbs are likely to be fundamentally different because 8B10 appears to be more sensitive to variations in protein conformation.

5F10 and 8B10 mAbs neutralize several CHIKV isolates, as well as ONN, but do not cross-react with other alphaviruses

It was critical to investigate the capacity of 5F10 and 8B10 to recognize and neutralize additional CHIKV isolates and other alphaviruses. Therefore, an immunofluorescence binding assay was performed with the following viruses: three additional CHIKV strains (from Singapore, Côte d’Ivoire, and Indonesia), ONNV, Semliki Forest virus, Ross River virus, Mayaro virus, Sindbis virus, Venezuelan equine encephalitis virus, and Eastern and Western equine encephalitis viruses. Both 5F10 and 8B10 display a strong reactivity with CHIKV strains from Singapore, Africa, and Indonesia, and with ONNV (Table II). However, they do not recognize any of the other alphaviruses evaluated.

We next assessed the ability of 5F10 and 8B10 to neutralize the three additional CHIKV strains and the ONN in a plaque reduction neutralization test. These viruses were completely neutralized by 100 μg/ml of either mAb (Fig. 5). However, the various isolates are not equally sensitive to neutralization at suboptimal mAb concentrations. In particular, both 5F10 and 8B10 efficiently neutralize the Singaporean and African CHIKV and ONNV, whereas the Indonesian CHIKV strain is poorly and hardly neutralized by 5F10 and 8B10, respectively.

Thus, 5F10 and 8B10 are highly specific for CHIKV. In addition, the 5F10 mAb may have a broader activity against different CHIKV isolates compared with the 8B10 mAb.

Discussion

This study describes the isolation and characterization of two human mAbs that specifically neutralize infection by several CHIKV strains in vitro.

The potency of 5F10 and 8B10 (IC50 < 100 ng/ml against the Singapore CHIKV11 isolate) is comparable or lower than those measured for other antiviral mAbs in similar neutralization tests.

### Table 1. Potency of anti-CHIKV mAbs against the Singapore CHIKV11 isolate

| mAb | (mean ± SD, μg/ml) | p Value
|-----|-------------------|--------
| IC50 | 5F10 | 8B10 | Combination |
| 0.062 ± 0.081 | 0.046 ± 0.026 | 0.038 ± 0.032 | NS |
| 0.932 ± 0.675 | 0.129 ± 0.028 | 0.100 ± 0.028 | NS |

*Data are from five independent plaque reduction neutralization tests.*

*The p values were calculated by Friedman’s test.*
For example, the anti-respiratory syncytial virus mAb palivizumab and the anti-coronavirus mAb CR3014 have an IC₅₀ of 2 μg/ml (29, 30), whereas the reported IC₅₀ of anti-influenza virus, anti-human CMV, and anti-West Nile virus mAbs range between 55 and 92 ng/ml (31–33). Therefore, the in vitro potency of 5F10 and 8B10 might be adequate for in vivo protection, which remains to be tested.

The broad coverage of multiple CHIKV strains is also a desired feature for an anti-CHIKV mAb to be a useful therapeutic tool. Three distinct CHIKV phylogroups have been identified: West African, Central/East African, and Asian (34, 35). The CHIKV strain that prevailed in Indian Ocean in 2005 and subsequently spread to India and Southeast Asia, including Singapore, was phylogenetically derived from the Central/East African CHIKV group (35–37). In terms of structural polyprotein homology, the Singapore CHIKV isolates used in this study are close to the 06-021 and Tanzanian S27 CHIKV strains (35), and may thus be classified within the Central/East African CHIKV group. The 5F10 and 8B10 mAbs neutralize Central/East African and West African (Côte d’Ivoire) CHIKV strains and, although much less efficiently, one Asian (Indonesia) strain. This was surprising because Asian and Central/East African CHIKV strains are phylogenetically closer to each other than to the West African group (34, 35).

5F10 and 8B10 are highly specific for CHIKV and do not recognize other members of the Alphavirus genus tested except the ONNV. This may be explained by the fact that CHIKV shares 85% of homology with the structural polyprotein of ONNV but only 44 to 62% with that of other alphaviruses (1, 38). Indeed, ONNV was initially thought to belong to the CHIKV group (39). However, serological differences, the existence of a different mosquito vector, as well as significant phylogenetic variations within the E1 protein, led to consideration of the two viruses as independent (34, 40).

Our data show that 5F10 is specific for CHIKV E2 protein, which is thought to be involved in cell receptor recognition for viral entry (2), and suggest that 8B10 might bind to E2, but do not rule out its possible specificity for E1 or the E1/E2 heterodimer. Despite the lack of synergistic effect between the two mAbs in neutralizing assays, we believe that the 5F10 and 8B10 epitopes are different, as indicated by first, the inability of 8B10 to bind to the recombinant sE2-SNAP protein, and second, its lower capacity to neutralize the Indonesian CHIKV isolate. Thus, the lack of synergy might be caused by steric hindrance between the mAbs.

Given their efficient CHIKV-neutralizing activity and ability to contain extracellular viral spreading, the 5F10 and 8B10 mAbs might be promising to treat human CHIKV infection, even though their anti-CHIKV potency needs to be further investigated and confirmed in vivo. Although it is costly and impractical to protect significant portions of a population with an Ab-based drug, these mAbs could be useful as targeted prophylactic measure within populations at high risk for severe disease, such as pregnant women, elderly, and individuals with prior joint diseases (16, 21). Moreover, several reports have described long-lasting, CHIKV-specific IgM despite the short viremia associated with CHIKV infection (41, 42). This long-term immune response, shown also for other alphaviruses, seems to correlate with chronic arthralgia/arthritis and might be caused by persisting viral Ags (43). In this context, CHIKV-specific mAbs might be useful not only to combat acute CHIKV infections, but to attenuate disease severity in patients suffering from long-lasting, CHIKV-associated arthritis.

![Figure 4](http://www.jimmunol.org/)  
**FIGURE 4.** 5F10 is specific for the E2 protein. A. Recognition of the viral proteins by 5F10 and 8B10 mAbs was investigated by Western blot on CHIKV-infected or uninfected cell lysates, and on CHIKV particles. Binding was evaluated using both CHIKV07 and CHIKV11 isolates from Singapore. 5F10 was used at 1 μg/ml, whereas 8B10 and the irrelevant IgG1 were used at 20 μg/ml; anti-CHIKV plasma was used at 1:250. B. Soluble recombinant E2 was immunoprecipitated with 3E4, 5F10, 8B10, or irrelevant IgG1 mAbs. The immunoprecipitated proteins were analyzed by Western blot using 3E4 mAb (5 μg/ml) as the detection Ab. C. CHIKV11-infected cell lysate was immunoprecipitated by 3E4, 5F10, 8B10, or irrelevant IgG1 mAbs. Analysis of immunoprecipitated proteins was performed as described in B. D. Recognition of the soluble E1 protein by 5F10 and 8B10 was examined by Western blot using mAbs at 10 μg/ml and, as a positive control, anti-CHIKV plasma at 1:100.

![Figure 5](http://www.jimmunol.org/)  
**FIGURE 5.** 5F10 and 8B10 mAbs neutralize several CHIKV isolates, as well as the ONNV. Percent virus neutralization by 100 μg/ml (open symbols) and 100 ng/ml (black symbols) of (A) 5F10 and (B) 8B10 mAb, as measured by a plaque reduction neutralization test.
Although the mechanisms of CHIKV-associated rheumatoid arthritis have not yet been elucidated, one case of CHIKV-associated autoimmune anti-nuclear Abs was recently reported (44); in addition, cross-reactivity of CHIKV-specific Abs with auto-antigens was one of the hypotheses suggested to explain the link between CHIKV infection and subsequent rheumatoid arthritis-like disease (42, 45). This potential self-recognition could be a concern for candidate therapeutics anti-CHIKV mAbs. When tested for their reactivity as rheumatoid factors or anti-nuclear Abs, however, both 5F10 and 8B10 mAbs resulted negative (data not shown).

In conclusion, the two CHIKV-neutralizing human mAbs described in this study might become useful therapeutic tools, to be used alone or in combination with nonspecific antiviral agents (46). They may also be useful to investigate CHIKV diversity and pathogenesis, and to identify neutralizing epitopes for vaccination strategies.

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Disclosures

S.L. is scientific adviser of Humalya, a Vivalis affiliate. L.W., J.P.-A., C.W., and A.N. are inventors in a pending patent.

References


