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

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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, broadly, 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: 3258–3264.

Chikungunya virus (CHIKV) belongs to the *Alphavirus* genus within the *Togaviridae* family and is transmitted to humans by *Aedes* mosquitoes. CHIKV has a positive-strand RNA genome encoding four nonstructural proteins (NSP1–4) and three structural proteins: the capsid, E1, and E2 envelope proteins (1, 2). Initially isolated in 1952 in Tanzania (3), CHIKV was reported for the first time in Asia in 1958 (4) and has since been associated with numerous outbreaks in the African and Asian continents. In 2005–2006, an unprecedentedly large epidemic of CHIKV affected several Indian Ocean islands before spreading to India and Southeast Asia (5). Moreover, in 2007, CHIKV emerged for the first time in Italy, a temperate area (6), and is now a worldwide infectious threat (7).

Unlike other arboviruses, most human CHIKV infections are associated with clinical symptoms, such as high fever, headaches, rash, myalgia, and arthralgia (3, 8–10). The disease is usually self-

limiting and resolves in 1–2 wk. However, joint pain can persist for several weeks or months, with up to 64% of patients reporting arthralgia 1 y post-CHIKV infection (9, 11–15). Advanced age, prior joint pain, and underlying osteoarthritis comorbidity were identified as risk factors for long-term rheumatic manifestations (16). Moreover, numerous cases of active and destructive CHIKV-associated rheumatoid arthritis have been reported (17–19). The vertical transmission of the virus has also been extensively investigated these last few years (20–22). Although CHIKV mother-to-fetus transmission appears to be extremely rare when the infection occurs early during pregnancy, up to 50% of the neonates experience development of CHIKV-associated clinical symptoms when mothers are infected shortly before delivery. These neonates are at high risk for severe encephalopathic complications, leading to disabilities or death (21).

As of 2010, there is no available vaccine or specific treatment against CHIKV infection (23). However, a virus-like particle vaccine, consisting of CHIKV envelope proteins expressed in a lentiviral vector, has been described to induce protection against CHIKV challenge in a monkey model. Interestingly, IgG isolated from the virus-like particle-vaccinated monkeys prevent CHIKV infection in mice, demonstrating the critical role of the humoral response in the control of CHIKV infection (24). Moreover, Couderc and collaborators (25) recently demonstrated that Igs isolated from plasma of CHIKV convalescent patients can efficiently prevent and cure CHIKV infection in mice. Altogether, these results strongly support the use of passive immunotherapy to control CHIKV infection.

Ab-based therapies constitute the only mean to provide immediate immunity against an infectious agent. Historically, hyperimmune sera or purified Igs have been extensively used for treating several viral diseases including measles, rabies, hepatitis A, and hepatitis B. However, serotherapies are potentially associated with serious adverse effects including anaphylactic shock (26, 27).

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Abbreviations used in this article: CHIKV, chikungunya virus; ONNV, O'nyong-nyong virus.

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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 alphaviruses 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-Invitrogen) and 2% ADCM (Dendritics). HEK293T (ATCC No. CRL-N268) and Vero cells (ATCC 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-Invitrogen).

Plasmids, Abs, and recombinant proteins

The expression plasmid pPMhIgG1 (gift from Dr. John Wu, Promab Biotechnologies) encodes the mouse Igk signal peptide and the human IgG1 C region (CH1-3), cloned between NheI and NotI restriction sites. A SalI restriction site is located downstream of the signal peptide sequence.

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

The recombinant CHIKV.sE2 was previously described (28). In this study, we used sE2-SNAP, consisting of CHIKV.sE2 fused to SNAP (Covalys BioSciences AG) followed by a hexa-histidin tag, and expressed in *Drosophila* S2 cells. The soluble sE2-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 SalI and NotI restriction sites, for expression in HEK293TPM1 cells.

Viruses

The two CHIKV isolates CHK/Singapore/07/2008 and CHK/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⁺ B cells was determined by flow cytometry (FACSCalibur, Becton Dickinson). PBMCs were seeded into 96-well plates at densities of 10, 30, or 500 B cells per well. B cells were activated by adding either 1 μg/ml mouse anti-human CD40 mAb HMY (Dendritics) and 10,000 lyophilized L4 cells expressing FcγRII (Dendritics), or 10,000 lyophilized L6 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 L 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 NheI-SalI and NheI-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 1 μg/ml 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 mAb were used as positive and negative control, respectively. The binding of anti-CHIKV Abs was detected 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 mAb 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:

$$\text{Percent neutralization} = \left[1 - \frac{\text{PFU}_{\text{mAb of interest (each concentration)}}}{\text{Mean PFU}_{\text{negative control (all concentrations)}}} \right] \%$$

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 IC₈₀. 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

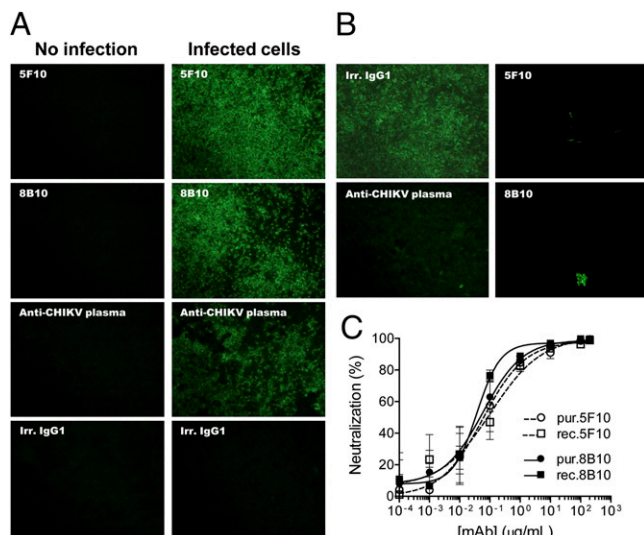


FIGURE 1. 5F10 and 8B10 mAbs bind and neutralize CHIKV *in vitro*. **A**, Purified mAbs bind the CHIKV11 isolate as assessed by immunofluorescence binding assay. Uninfected (*left panels*) or infected (*right panels*) HEK293T cells probed with 5F10, 8B10, anti-CHIKV plasma 1:200, or irrelevant IgG1. All mAbs were used at 1 μg/ml. **B**, Purified mAbs neutralize the CHIKV11 isolate in an immunofluorescence neutralization assay. Shown are also, as a positive control, anti-CHIKV plasma 1:20 and, as a negative control, irrelevant IgG1. All mAbs were used at 1 μg/ml. **C**, Recombinant mAbs are as potent as the corresponding purified mAbs in neutralizing the CHIKV11 isolate. mAbs were tested in a plaque reduction neutralization test across a concentration range from 100 pg/ml to 200 μg/ml. The means and SD from three independent matched experiments, as well as the nonlinear regression fitting curves, are shown. Original magnification ×100.

DMEM was added. Two and 6 h later, the number of PFU in the cell supernatant was determined by plaque assay. The percentage of neutralization was calculated as described earlier.

Immunoprecipitation and Western blot

For cell lysates preparation, 12 × 10⁶ CHIKV-infected or noninfected Vero cells were lysed with 1 ml PBS-1% Triton X-100 supplemented with Complete Protease Inhibitor Cocktail (Roche).

Viral particles were purified from 30 ml supernatant of 8 × 10⁷ CHIKV-infected Vero cells; virus was concentrated on Vivaspin 100-kDa columns (Sartorius Stedim), inactivated for 1 h at 56°C, and purified by ultracentrifugation (24,000 rpm for 3 h). Viral particles were resuspended in 1 ml PBS.

For immunoprecipitation, 2 μg sE2-SNAP or 120 μl cell lysate was incubated overnight with 10 μg Abs, then for 4 h with protein G agarose beads. Bound Abs/proteins were eluted with glycine (130 μl final volume).

Cell lysates (15 μl), purified viral particles (1.5 μl), CHIKV E1 (200 ng), sE2-SNAP (200 ng), or cell lysate immunoprecipitates (20 μl) were heated at 95°C for 5 min with or without NuPAGE Sample Reducing Agent (Invitrogen), separated by electrophoresis (NuPAGE 4–12% Bis-Tris Gel; Invitrogen) and electrotransferred onto polyvinylidene difluoride membranes (Hybond-P; Amersham, GE Healthcare). The membranes were incubated with mAbs or anti-CHIKV plasma, followed by peroxidase-

conjugated goat anti-human or anti-mouse IgG. Peroxidase activity was detected using ECL substrate solutions (Amersham ECL Western blotting Detection Reagents; GE Healthcare).

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 IgG1λ2 and IgG1κ, 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; IC₅₀ and IC₈₀ (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: IC₅₀ ranged between 10 and 200 ng/ml, and IC₈₀ 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 were infected with CHIKV11, then treated for 2 h with different concentrations of mAbs. The amount of infective CHIKV11 particles present in the media was determined 2 and 6 h

FIGURE 2. Recombinant 5F10 and 8B10 mAbs have comparable potency and are not synergistic *in vitro*. **A**, mAbs were tested as single agents or in combination using a plaque reduction neutralization test across a concentration range from 100 pg/ml to 200 μg/ml. The mean percent neutralization and SD from five independent matched experiments, as well as nonlinear regression fitting curves, are shown. **B**, IC₅₀ (*left panel*) and IC₈₀ (*right panel*) values extrapolated from five independent experiments with the single mAbs and the combination. Also shown is the mean (see Table I for values).

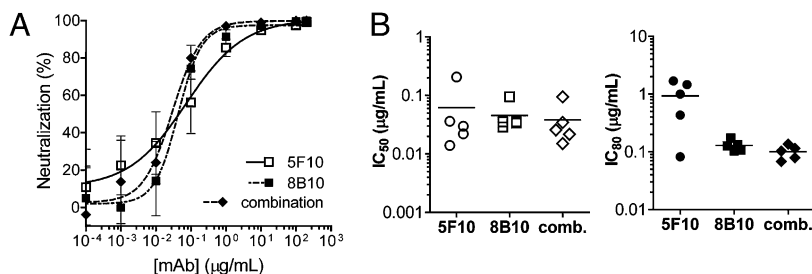


Table I. Potency of anti-CHIKV mAbs against the Singapore CHIKV11 isolate

	Abs (mean \pm SD, $\mu\text{g/ml}$) ^a			<i>p</i> Value ^b
	5F10	8B10	Combination	
IC ₅₀	0.062 \pm 0.081	0.046 \pm 0.026	0.038 \pm 0.032	NS
IC ₈₀	0.932 \pm 0.675	0.129 \pm 0.028	0.100 \pm 0.028	NS

^aData are from five independent plaque reduction neutralization tests.

^bThe *p* values were calculated by Friedman's test.

post-treatment. After 2 h, 1 $\mu\text{g/ml}$ 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 of 100 $\mu\text{g/ml}$ 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 recognized a 49-kDa band from both CHIKV particles and CHIKV-infected cells lysates, although recognition by 8B10 was much weaker than by 5F10 (Fig. 4A). The two CHIKV envelope glycoproteins, E1 and E2, form a heterodimer at the viral surface, and because of a similar molecular mass of \sim 50 kDa, they cannot be distinguished by Western blot. These results therefore indicate that 5F10 and 8B10 mAbs bind to E1, E2, or both. Moreover, 5F10 recognized a 30-kDa polypeptide in CHIKV-infected cell lysates (Fig. 4A). The polypeptide was also reactive with the CHIKV E2-specific mouse mAb 3E4 (data not shown). Thus, it is likely that the 30-kDa polypeptide is an antigenic fragment derived from the CHIKV E2 protein.

The recombinant sE2-SNAP was then used to determine more precisely the mAb antigenic specificity. 5F10, but not 8B10, was capable of immunoprecipitating sE2-SNAP (Fig. 4B), demonstrating that 5F10 binds to E2. 8B10 could be specific for E1 or for the E1/E2 heterodimer; alternatively, 8B10 might be specific for E2 but unable to bind to the recombinant sE2-SNAP protein because of potential conformational differences compared with the native CHIKV E2. Thus, we next performed an immunoprecipitation from lysate of CHIKV11-infected cells, containing native forms of E1 and E2. Fig. 4C shows that 8B10, like 5F10,

immunoprecipitates 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 coimmunoprecipitation 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 $\mu\text{g/ml}$ 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.

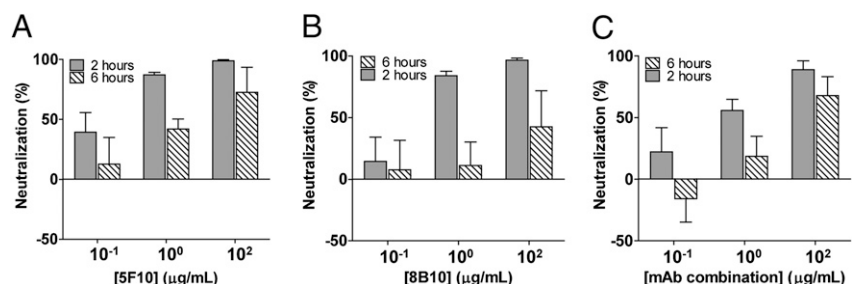
Thus, 5F10 and 8B10 are highly specific for CHIKV. In addition, the 5F10 mAb may have a broader activity against different CHIKV isolates compared with the 8B10 mAb.

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.

FIGURE 3. 5F10 and 8B10 mAbs are capable of limiting extracellular viral spreading in a post-infection neutralization assay. Eleven hours after CHIKV11 infection, infected HEK293T cells were treated for 2 h with recombinant mAbs as single agents (A, B) or in combination (C) at concentrations ranging from 100 ng/ml to 100 $\mu\text{g/ml}$. Viral titer in the supernatant was measured 2 and 6 h after treatment. Shown are mean percent neutralization and SD from three independent experiments.



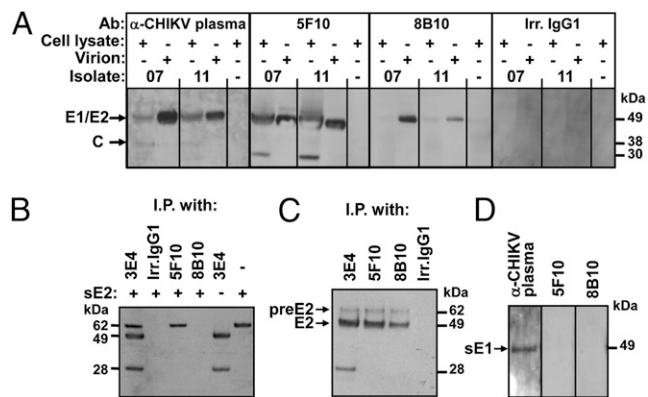


FIGURE 4. 5F10 is specific for the E2 protein. *A*, Recognition of the viral proteins by 5F10 and 8B10 mAbs was investigated by Western blot on CHIKV-infected or uninfected cell lysates, and on CHIKV particles. Binding was evaluated using both CHIKV07 and CHIKV11 isolates from Singapore. 5F10 was used at 1 μ g/ml, whereas 8B10 and the irrelevant IgG1 were used at 20 μ g/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 μ g/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 μ g/ml and, as a positive control, anti-CHIKV plasma at 1:100.

For example, the anti-respiratory syncytial virus mAb palivizumab and the anti-coronavirus mAb CR3014 have an IC_{50} of 2 μ g/ml (29, 30), whereas the reported IC_{50} 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

Table II. Cross-reactivity of anti-CHIKV mAbs with other alphaviruses

Virus ^a	Abs		
	Antivirus Ascite ^b	5F10 ^c	8B10 ^c
Singapore CHIKV07	NA	+	+
Côte d'Ivoire CHIKV	+	+	+
Indonesia CHIKV	+	+	+
ONNV	+	+	+
Ross River virus	+	–	–
Semliki Forest virus	+	–	–
Mayaro virus	+	–	–
Sindbis virus	+	–	–
Venezuelan equine encephalitis virus	+	–	–
Eastern equine encephalitis virus	NA	–	–
Western equine encephalitis virus	NA	–	–

^aVero cells were infected with the indicated viruses and fixed 24 h postinfection. A binding assay was performed by immunofluorescence using, as a secondary Ab, either an anti-mouse IgG conjugated to Alexa 488 (*b*) or an anti-human IgG conjugated to Alexa 488 (*c*).

^bMouse immune ascites specific for each indicated virus were used 1/200.

^cmAbs were used at 1 μ g/ml.

+, strong fluorescence signal; –, no fluorescence signal; NA, not assessed.

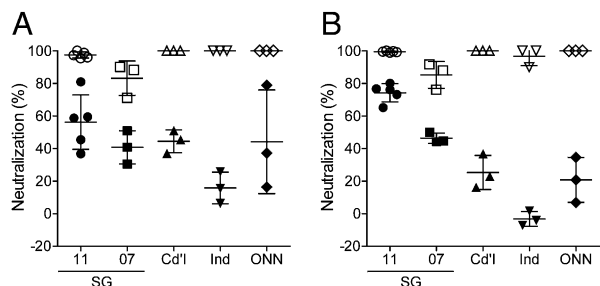


FIGURE 5. 5F10 and 8B10 mAbs neutralize several CHIKV isolates, as well as the ONNV. Percent virus neutralization by 100 μ g/ml (open symbols) and 100 ng/ml (black symbols) of (*A*) 5F10 and (*B*) 8B10 mAb, as measured by a plaque reduction neutralization test.

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 unpractical 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.

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 Humalys, a Vivalis affiliate. L.W., J.-P.A., C.W., and A.N. are inventors in a pending patent.

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