A potent neutralizing IgM mAb targeting the N218 epitope on E2 protein protects against Chikungunya virus pathogenesis

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Chikungunya virus (CHIKV) is a medically important human viral pathogen that causes Chikungunya fever accompanied with debilitating and persistent joint pain. Host-elicited or passively-transferred monoclonal antibodies (mAb) are essential mediators of CHIKV clearance. Therefore, this study aimed to generate and characterize a panel of mAbs for their neutralization efficacy against CHIKV infection in a cell-based and murine model.

To evaluate their antigenicity and neutralization profile, indirect enzyme-linked immunosorbent assay (ELISA), an immunofluorescence assay (IFA) and a plaque reduction neutralization test were performed on mAbs of IgM isotype. CHIKV escape mutants against mAb 3E7b neutralization were generated, and reverse genetics techniques were then used to create an infectious CHIKV clone with a single mutation. 3E7b was also administered to neonate mice prior or after CHIKV infection. The survival rate, CHIKV burden in tissues and histopathology of the limb muscles were evaluated. Both IgM 3E7b and B2A2c bind strongly to native CHIKV surface and potently neutralize CHIKV replication. Further analyses of 3E7b binding and neutralization of CHIKV single-mutant clones revealed that N218 of CHIKV E2 protein is a potent neutralizing epitope. In a pre-binding neutralization assay, 3E7b blocks CHIKV attachment to permissive cells, possibly by binding to the surface-accessible E2-N218 residue. Prophylactic administration of 3E7b to neonate mice markedly reduced viremia and protected against CHIKV pathogenesis in various mice tissues. Given therapeutically at 4 h post-infection, 3E7b conferred 100% survival rate and similarly reduced CHIKV load in most mice tissues except the limb muscles. Collectively, these findings highlight the usefulness of 3E7b for future prophylactic or epitope-based vaccine design.

Introduction

Chikungunya virus (CHIKV) is a medically important human alphavirus known to cause distinctive polyarthritis or polyarthralgia.1 Common clinical features include fever, maculopapular rash, myalgia,2 while severe complications such as encephalitis and mortality may occur in immunocompromised individuals,3 the elderly4 and infants.5,6 As a human arbovirus, CHIKV is transmitted by Aedes aegypti and Aedes albopictus mosquitoes. Since 2004, explosive epidemics in Africa,7 Indian Ocean islands8 and India9 have propelled CHIKV dissemination to various non-endemic countries in South-East Asia,10 Australia,11 Europe and USA.12,13 At present, millions of CHIKV infection cases have been reported worldwide and virus transmission remains active in various Caribbean countries,14 thus signaling the risk of an imminent global CHIKV epidemic.

CHIKV has a positive-sense RNA genome that encodes 4 non-structural proteins (nsP1, 2, 3, 4), 3 structural proteins (capsid, envelope glycoprotein E1 and E2) and 2 cleavage products (E3 and 6k).15 Structurally, the mature E2 protein adopts 3 immunoglobulin-like folds known as domain A at the N-terminal, domain B at the tip and domain C at the C-terminal, which is closest to the viral membrane. The latter is followed by a stem-like transmembrane helix and cytoplasmic tail.16 The extracellular ectodomain comprising domain A, B and C are interconnected by beta-ribbon. Through extensive array of hydrogen bonds, salt bridges and van der Waals forces, E2 intrinsically complexed with E1 protein to form heterodimer that arranged as 80 trimeric spikes on the viral lipid envelope.16,17 With such a delicate virion surface architecture, E1 and E2 participate complementarily in CHIKV entry. As a type-I transmembrane protein, E2 first mediates CHIKV attachment to the cellular receptor by interaction with surface-exposed regions on domain A and B.18 E1, being a type-II fusion protein, subsequently promotes viral membrane fusion within acidified endosomal membrane to release CHIKV nucleocapsid into the host cytosol.19

Currently, there are no licensed vaccine or effective antiviral for CHIKV disease. Available treatments based on nonsteroidal
anti-inflammatory drugs, analgesics or a combination of corticosteroids are symptomatic, associated with side effects and ineffective for CHIKV-induced chronic arthritis or neonatal infection from viremic mother. A plethora of studies have evaluated chemical compounds and antisense agents as potential CHIKV antivirals, but these therapies may not achieve favorable pharmacosafety and tissue-targeted delivery in vivo. In contrast, vaccination strategies have highlighted the importance of humoral immunity in controlling CHIKV infection. Strong long-lasting mAb-mediated protection in infected individuals and animal models was observed after administration of CHIKV-based vaccines. Passive transfer of anti-CHIKV mAbs purified from the convalescent serum of infected patients or co-administration of pairs of neutralizing mAbs to interferon receptor (IFNR)-deficient mice model was shown to confer significant therapeutic and prophylactic efficacy.

Some of the neutralizing mAbs identified were also conserved in their efficacy against several CHIKV isolates of different genotypes. Altogether, these studies emphasized mAbs as a promising antiviral strategy for CHIKV infection at both pre- and post-exposure settings.

To characterize mAb binding ability to CHIKV, indirect IFA and indirect virion-based ELISA were carried out. Notably, 3E7b and 8A2c IgM strongly detected CHIKV antigen intracellularly, likely due to being in close association with the endoplasmic reticulum (ER) during active virus replication as well as on the plasma membrane of CHIKV-infected cells at day 1 p.i. (Fig. 1A; arrowheads). These observations were consistent with the staining by mAb positive control using our laboratory-established rabbit anti-CHIKV E2 polyclonal IgG. Lack of strong staining was seen in all mock-infected cells, thereby suggesting that these mAbs are specific to CHIKV antigen. To further decipher whether 3E7b and 8A2c binds directly to native surface of CHIKV and neutralize CHIKV infection potently with IC50 of 4–5 ng/ml. Additionally, 3E7b showed no cross-reactivity to other related alphaviruses, the Ross River virus (RRV) and Sindbis virus (SINV). Sequence analysis of 3E7b escape mutants identified 3 distinct mutations that are solvent-accessible and sequence-conserved on CHIKV E2 protein. However, further studies through reverse genetics identified only E2-N218 as critical for 3E7b binding and neutralization. Prophylactic evaluation of 3E7b in a neonate mouse model revealed a dose-dependent efficacy in prolonging survival, as well as significant protection of CHIKV-induced muscle pathogenesis. When administered 4 h post-CHIKV challenge, 3E7b similarly prolonged survival and reduced CHIKV burden in various tissues except the limb muscles, thus suggesting that it could be less effective in a therapeutic setting. Taken together, our findings can be projected for a rationale epitope vaccine design.

Results

Anti-CHIKV IgM 3E7b and 8A2c bind strongly to surface of native CHIKV virion

In this study, adult BALB/c mice were challenged with whole CHIKV virion to elicit a wide repertoire of antibodies that can recognize the viral envelope glycoprotein, E1 and E2. After the third immunization at 28 day post-infection (p.i.), mice sera were harvested and analyzed in Western blot for antibody detection of CHIKV proteins from CHIKV-infected BHK-21 cell lysates. A 35-38 kDa band, suggestive of CHIKV capsid protein was strongly detected by all sera, while a weak band of 52–55 kDa likely to be E1 or E2 protein was recognized by mice sera 1 to 4 (Fig. S1A). This indicates the presence of CHIKV-specific antibody response following the acute phase of CHIKV infection in mice. The spleen of mouse 1 was subsequently harvested for the production of anti-CHIKV mAbs. Culture supernatant from hybridoma clones were screened by immunofluorescence neutralization assay on BHK cells infected with CHIKV at MOI 10. Among the 30 successful subclones, 4 clones, 3E7b, 8A2c, 8D3d and 10C5b, were shown to neutralize CHIKV infection where at least 90% of cells were stained negatively for CHIKV antigen (Fig. S1B–C). Based on this result, these clones were expanded by production of mice ascites and individual ascites of 3E7b and 8A2c clones were further purified and identified as IgM mAbs.

To characterize mAb binding ability to CHIKV, indirect IFA and indirect virion-based ELISA were carried out. Notably, 3E7b and 8A2c IgM strongly detected CHIKV antigen intracellularly, likely due to being in close association with the endoplasmic reticulum (ER) during active virus replication as well as on the plasma membrane of CHIKV-infected cells at day 1 p.i. (Fig. 1A; arrowheads). These observations were consistent with the staining by mAb positive control using our laboratory-established rabbit anti-CHIKV E2 polyclonal IgG. Lack of strong staining was seen in all mock-infected cells, thereby suggesting that these mAbs are specific to CHIKV antigen. To further decipher whether 3E7b and 8A2c binds directly to native surface of CHIKV, purified CHIKV was coated onto ELISA plate and a range of mAbs was added. Both 3E7b and 8A2c showed dose-dependent binding to CHIKV, consistent with the mAb positive control, mouse anti-CHIKV E2 IgG. Binding of 3E7b and 8A2c to CHIKV are close to saturation at higher than 25 ng of IgM.

Taken together, the results indicate 3E7b and 8A2c IgMs are highly specific to CHIKV antigen, particularly to the native E1 or E2 protein exposed on the surface of CHIKV.

Anti-CHIKV IgM specifically and strongly neutralizes CHIKV

To evaluate if there is a dose-dependent neutralizing efficacy of mice ascites and purified IgMs against CHIKV, an in vitro Plaque Reduction Neutralization Test (PRNT) was optimized
for this purpose. In brief, mice ascites or purified IgMs were serially-diluted in sterile phosphate-buffered saline (PBS) before each dilution was incubated with 100 plaque-forming units (PFU) of CHIKV for 1 h at 37°C. The mixture was then added to confluent BHK-21 cells. Consistent with results from the earlier immunofluorescence neutralization assay screening, mice ascites of 3E7b, 8A2c and 10C5b clones strongly inhibited CHIKV replication (Fig. 2A). In particular, 3E7b ascites remained effective at a high dilution of 1:20,000. In contrast, 8D3d ascites failed to be neutralizing even though it showed strong efficacy in previous neutralization screening. As the exact IC₅₀ could not be determined from mice ascites test, PRNT was further performed on purified IgM. Both the purified 3E7b and 8A2c are strongly neutralizing against 100 PFU of CHIKV across the wide range of mAb concentration tested (Fig. 2B). Based on the dose-dependent neutralization curves obtained, 3E7b has an IC₅₀ of 4.47 ng/ml while 8A2c has IC₅₀ of 3.99 ng/ml, suggesting that 8A2c is slightly more neutralizing than 3E7b (Fig. 2C). Taken together, both 3E7b and 8A2c were remarkably potent against CHIKV replication.

To elucidate the plausible mechanism of neutralization, a post-PRNT was performed to determine if the neutralization efficacy is maintained when 3E7b was added after CHIKV has bounded to the cell surface. CHIKV of 100 PFU was added to BHK cells at 4°C to allow virus binding for 1 h, followed by incubation with 3E7b for 30 min at 4°C. Subsequently, PRNT was performed as described earlier. Here, it is notable that 3E7b failed to block CHIKV replication after virus-host cell attachment has occurred (Fig. 2D). This suggests that 3E7b-mediated neutralization of CHIKV requires a pre-incubation of the mAb with CHIKV, probably to allow its binding to the viral E2 epitopes to form 3E7b-CHIKV complex. Therefore, this leads to ineffective CHIKV attachment when the mixture is added to BHK cells.

Next, we investigated whether 3E7b could have a broad neutralizing efficacy against other CHIKV strain or alphaviruses. Here, the standard PRNT was performed by incubating 3E7b with 100 PFU of CHIKV Ross strain, Sindbis virus (SINV) or Ross River virus (RRV), respectively. SINV is a representative member of the Sindbis serocomplex while RRV shares the same Semliki Forest Virus serocomplex with CHIKV. Relative to the IgM isotype control, 3E7b neutralized CHIKV Ross dose-dependently, but no evident neutralization was observed against SINV and RRV replication (Fig. 3A–B). This suggests that 3E7b is
specific in CHIKV neutralization. However, compared to CH122508 strain (Fig. 2B), 3E7b was less effective in neutralizing CHIKV Ross replication. Though both CH122508 and Ross strains belong to the same ECSA lineage, differences in the 3E7b neutralizing epitopes may contribute to the lower potency against CHIKV Ross replication.

CHIKV escape mutants against 3E7b neutralization contained 3 mutations in viral E2 protein

To identify critical amino acid residues required for CHIKV neutralization, escape mutant virus against 3E7b was generated. CH122508 strain was sequentially passaged in BHK cells for 10 rounds under increasing selective pressure from 3E7b, followed by 2 additional rounds of virus plaque-purification to isolate individual escape clones. In parallel to 3E7b, CHIKV was also passaged in PBS (the absence of mAb as mock selection) and in the presence of IgM isotype (as IgM specificity control). A total of 69 escape clones, namely 57 CHIKV/3E7b clones, 8 CHIKV/IgM clones and 4 CHIKV/PBS clones were obtained. When representative escape clones were further evaluated in IFA, it was shown that CHIKV selected from 3E7b neutralization abolished binding to 3E7b while CHIKV selected from IgM isotype and PBS treatment retained binding ability to 3E7b (data not shown). This implies a likely escape mutation on CHIKV protein that conferred resistance to 3E7b binding.

To further assess if this mutation was associated with a loss of neutralization, a representative escape clone CHIKV/3E7b 1a was analyzed in PRNT. Both CHIKV/PBS 1a and CHIKV/IgM 1a clones were susceptible to 3E7b neutralization with IC_{50} of 0.12 μg/ml and 0.17 μg/ml, respectively (Fig. 4A). However, CHIKV/3E7b 1a clone was not neutralized by 3E7b at 2.5 μg/ml, the highest concentration used earlier for the generation of escape mutation. Viral RNA from CHIKV/3E7b escape clones were reverse-transcribed and sequenced for the entire length of viral E1 and E2 gene. Notably, all 7 representative CHIKV/3E7b clones showed 3 single point mutation in E2 gene that results in E \$$\Rightarrow$$ D at residue 24 (E24D), N \$$\Rightarrow$$ D at residue 218 (N218D) and D \$$\Rightarrow$$ G at residue 223 (D223G) in the E2 protein (Fig. 4B). No mutation was identified in CHIKV/PBS 1a, CHIKV/IgM 1a, CHIKV/IgM 4a and the wildtype CH122508 strain E2 sequence, thereby suggesting that escape mutations are specific only to CHIKV that has been selected under the neutralizing pressure of 3E7b. In addition, none of the escape clones, represented by CHIKV/3E7b 3a and 7a clones, as well as CHIKV/IgM 4a and CHIKV/PBS 1a control clones have any mutation in E1 gene (data not shown). All E2 mutations are single amino acid substitutions that did not cause translational frameshift and CHIKV escape mutants remained detectable by a positive mAb control, the anti-CHIKV E2 IgG in IFA (data not shown). Taken together, the escape mutations did not cause any global effect on E2 conformation.

Various studies have demonstrated that an escape mutation on viral envelope protein represents a critical mAb epitope for virus neutralization. Using the crystal structure of mature E1/E2 glycoprotein complex (PDB: 3N43) of CHIKV, we mapped the 3 identified escape mutations onto this model. E24 is found to
be located on the N-terminal hairpin of domain A while N218 and D223 are both located on the outermost tip of domain B (Fig. 4C). Collectively, these residues are positioned on random linker coils between the beta sheets of domain A and B. Relative to E24, both N218 and D223 are spatially closer to each other, with a proximity of 13.0 Å. Hence, N218 and D223 could constitute the epitope for 3E7b binding to the native conformation of CHIKV E1/E2 heterodimer. Notably, all 3 residues are also highly surface accessible, with estimated values of 89.9%, 69.2%, 77.6%, respectively. This suggests that 3E7b can freely bind to these epitopes on the native virion without much steric hindrance. Given its high surface accessibility, it is also favorable for these mutations, E24D, N218D and D223G, to occur for escape neutralization from 3E7b.

E2-N218D mutation confers CHIKV resistance to 3E7b binding and neutralization

In the interest of investigating the functional role of individual E2 mutation in mediating escape from 3E7b neutralization, reverse genetics technique was employed to generate full-length infectious CHIKV clones that contained a single mutation of E24D, N218D or D223G. These single mutant clones are named as CHIKV-E24D, CHIKV-N218D, CHIKV-D223G. Together with CHIKV-IVT (CHIKV generated from in vitro transcribed-infectious clone cDNA template) and CHIKV-WT (wildtype CH122508 strain), they were evaluated for their replication competency in BHK cells. Viral supernatant were collected at specific timepoint p.i. and quantitated by plaque assay. All CHIKV mutant clones demonstrated similar growth kinetic profile to CHIKV-IVT and CHIKV-WT virus controls (Fig. S2A). Collectively, the virus titer rapidly increased within the first 36 h p.i. and infectious cDNA clone-generated CHIKV displayed higher replication efficacy relative to CHIKV-WT control. Among these clones, minor differences in the peak of virus production were also observed. However, there were no evident differences in the plaque size and morphology of the mutant clones relative to CHIKV-IVT and wildtype controls at 24 h p.i (Fig. S2B). Taken together, these data suggest that the engineered mutation in viral E2 protein did not greatly affect mutant virus replication or extracellular spreading of progeny virus in BHK cells.

Next, we investigated if the single, surface-exposed mutation on CHIKV E2 protein could affect virus binding to 3E7b. A dual-color IFA assay was performed on BHK cells-infected with CHIKV-E24D, CHIKV-N218D and CHIKV-D223G at MOI 10, respectively. CHIKV E2 binding to 3E7b was tested by co-staining of viral-infected cells with 3E7b IgM and anti-CHIKV E2 IgG positive control followed by quantification of the respective signals. Interestingly, the binding of CHIKV-N218D to 3E7b was significantly reduced where 3E7b-FITC signal was absent on the cell membrane and only some diminished signal was seen in the cytosol of the infected cells at day 1 p.i. (Fig. 5A; white arrowheads). On the contrary, CHIKV-E24D and CHIKV-D223G retained strong binding efficacy to 3E7b similar to CHIKV-WT and CHIKV-IVT controls. Comparatively, only 2.1% of the cells infected with CHIKV-N218D were positively stained with 3E7b relative to 95.3% and 99.5% binding in CHIKV-WT and CHIKV-IVT, respectively (Fig. 5B). In the latter CHIKV control-infected cells, colocalized signal arising from bound 3E7b-FITC and anti-E2 594 was consistently observed in the ER and plasma membrane. This indicates intracellular CHIKV E2 synthesis at the ER and localization of CHIKV E2 at the plasma membrane for progeny virus budding. From these observations, only N218D mutation, but not E24D and D223G, is critical for 3E7b binding.

Poor binding of 3E7b to CHIKV due to E2-N218D mutation suggests that N218 could be a critical neutralizing epitope. To validate this hypothesis, PRNT was performed using 0.25 μg/ml of 3E7b, the neat concentration optimized previously. Respectively IC_{50} values of mutant clones were calculated by...
non-linear regression analysis and compared to CHIKV-WT and CHIKV-IVT controls. Evidently, CHIKV-N218D was able to confer resistance to 3E7b neutralization, whereas CHIKV-E24D and CHIKV-D223G mutants were neutralized effectively over the tested range of 3E7b concentration from 3.81 pg/ml to 0.25 μg/ml (Fig. 5C). Relative to CHIKV-WT, the IC₅₀ values of 3E7b from neutralization of CHIKV-IVT, CHIKV-E24D and CHIKV-D223G are at least 2.6-fold higher. Decreased neutralization efficiency could be due to inherent differences in infectious-clone generated CHIKV clones. Nonetheless, the overall neutralization trend was similar among CHIKV-WT, CHIKV-IVT and mutant clones except for CHIKV-N218D.

In order to determine if N218 vary among different lineages of CHIKV, sequences flanking the mutation were aligned with all existing CHIKV structural polyprotein and E2 sequences in GenBank and PDB. These escape mutations were found to be 100% identical with >178 published sequences. In this alignment, high sequence homology is also shared between different CHIKV genotypes, namely East/Central/South African (ECSA), Asian and West Africa lineage (data not shown). Therefore, this implies that the conserved escape residues on E2 could be critical for CHIKV replication across different genotypic strains. Taken together, our findings validate that N218 residue is highly conserved on CHIKV E2, and it is an important epitope for potent neutralization by mAb 3E7b.

Prophylactic administration of 3E7b protects mice from CHIKV disease

Having shown that 3E7b was potently neutralizing in vitro, it is necessary to investigate if the mAb can similarly exert antiviral efficacy in vivo. Herein, 3E7b was tested in a neonate mouse model of CHIKV infection that was previously well-established in our laboratory. In this model, 6-day old BALB/c mouse was shown to be highly susceptible to CHIKV infection, specifically to CH6708 strain (LK(EH))
CH6708, GenBank Accession number: FJ513654). As they have yet to develop a mature innate immunity, this model displayed clinical features similar to those reported in CHIKV-infected patients.39,40 This suggests that neonate mouse is an ideal in vivo model for recapitulating CHIKV pathogenesis. Based on our previous laboratory findings, CHIKV infection was strain-specific in our neonate mouse model where mice succumb only to CH6708 infection with CHIKV disease manifestations. Indeed, we observed that during the acute phase of CHIKV infection at day 2–4 p.i., neonate mice developed high viremia and viral loads in various tissues including the hind limb muscles, liver, spleen and brain (data yet to be published). High CHIKV load is accompanied with an onset of hind limb paralysis at day 3–4 p.i., which is characteristic of CHIKV disease, followed by lethality within day 5–9 p.i. Because CH6708 strain shares a high 99.7% genomic similarity with CH122508 strain, it is considered identical to CH122508 strain for testing of 3E7b efficacy in vivo. Using this 6-day old BALB/c mouse model, the prophylactic efficacy of 3E7b was assessed over a mAb dose range of 0.2, 2, 20 or 200 μg per mouse that was given intraperitoneally at 24 h and 8 h prior to infection with 4 × 10^5 PFU of CH6708 strain. IgM isotype antibody and PBS (mock-mAb) were included as irrelevant mAb treatment and diluent control, respectively. All control mice that received PBS succumbed to lethal CHIKV infection by day 9 p.i., while IgM isotype-treated mice showed poor survival (40%) at day 14 p.i. (Fig. 6A). However, mice pre-treated with 3E7b were able to overcome CHIKV-induced lethality, with a good baseline level of 80% survival across the given doses. Dose-dependent protection is highly significant by log-rank test (p < 0.001), with 80% survival in 0.2 μg and 2 μg 3E7b treatment groups and 100% survival in 20 μg and 200 μg treatment. To further assess the minimum prophylactic dose required during the acute phase of CHIKV infection, mice were given the same treatment regimen, sacrificed on day 2 p.i. and virus titer in the serum and various tissues were quantitated. Relative to PBS-infected control, pre-treatment with 2 μg of 3E7b significantly cleared CHIKV burden in serum (Fig. 6B) and other virus-targeted tissues, namely, limb muscles (Fig. 6C) (p < 0.05), brain (Fig. 6D) (p < 0.001), liver (Fig. 6E) and spleen (Fig. 6F) (p < 0.01).
Dose-dependency is also consistently observed where 0.2 μg of 3E7b pre-treatment failed to confer much protection against CHIKV infection in these mice tissues.

Having shown that 3E7b effectively reduced CHIKV load in limb muscles at day 2 p.i., we further examined if the protective efficacy of 3E7b can be sustained in the skeletal muscles during the course of CHIKV infection. Mice hind limbs were harvested at day 7 p.i., paraformaldehyde-fixed and processed for Hematoxylin and Eosin (H&E) staining. The skeletal muscles of PBS-treated mice showed extensive necrosis with loss of striations and infiltration of inflammatory cells (Fig. 7). IgM isotype-treated mice showed less severe muscle necrosis and inflammation relative to PBS control. In contrast, hind limb muscles of 3E7b-treated mice retained healthy striated morphology as seen by absence of abnormality or necrosis. Therefore, pre-treatment of 3E7b could have prevented CHIKV dissemination to muscle tissues and thereby protects against CHIKV-induced muscle pathogenesis.

**Therapeutic administration of 3E7b protects mice from CHIKV disease**

Based on the findings from prophylactic treatment, consecutive doses of 20 μg 3E7b were effective in preventing CHIKV-induced lethality and limb muscle pathology. Therefore, it is interesting to assess if the same dose could also protect mice from CHIKV infection when given at post-CHIKV exposure. Using the same neonate mouse model, 6-day old mice were infected with $4 \times 10^5$
10^5 PFU of CHIKV prior to receiving a single 20 µg dose of 3E7b at 4 h or 8 h p.i. Mice given PBS diluent or IgM isotype showed poor survival (less than 20%; Fig. 8A). For mice post-treated with 3E7b at 8 h p.i., survival was improved to 50% (p < 0.05). However, when given at 4 h p.i., 3E7b was significantly effective (p < 0.01), protecting all mice against CHIKV-induced lethality. This suggests a time-dependent neutralizing efficacy of 3E7b during acute infection where there is high CHIKV burden.

With a defined 3E7b therapeutic window at 4 h p.i., we further evaluated the level of CHIKV load in the serum and various mouse tissues at day 2 p.i. Relative to PBS-infected control, 3E7b was significantly effective (p < 0.01) in reducing viral loads in the serum (Fig. 8B), liver (Fig. 8E) and spleen (Fig. 8F), while a lower significance is observed in the brain tissue (Fig. 8D) (p < 0.05). There was at least a 4-log decrease in CHIKV titer consistently in these tissues. However, a high level of CHIKV load was consistently detected in the hind limb muscles (Fig. 8C).

Histological analysis of mice limb muscles at day 7 p.i. revealed partial protection, where some areas of the muscles retained healthy morphology (Fig. 9) while some areas were evidently necrotized with infiltration of inflammatory cells. Despite such muscle pathology, these mice did not display limb paralysis and were able to survive against CHIKV infection over 14 days p.i. Taken together, this suggests that the protective effect of 3E7b against CHIKV infection in other tissues could have helped to overcome CHIKV-induced lethality. Therefore, these data demonstrate that therapeutic administration of 20 µg 3E7b was protective in most CHIKV-targeted tissues and could improve the overall CHIKV disease outcome.

**Discussion**

At present, neutralizing CHIKV-specific mAbs that were characterized and reported belong to IgG isotype, and they were either produced in mice by a combination of passive immunization and infectious CHIKV challenge^{29,31} or derived from the sera of patients.^{30,33,34} In this study, we inoculated live CHIKV in mice to generate envelope protein-specific mAbs that could confer strong neutralization potency. From PRNT analysis, 2 of our anti-CHIKV IgMs, 3E7b and 8A2c, demonstrated strong neutralization potency of 4-5 ng/ml in CHIKV-infected BHK cells. The most potent anti-CHIKV mAb previously described, CHK-152 IgG, has an in vitro IC_{50} of 1-3 ng/ml in Vero and mouse fibroblast cells.^{29} Because IgM is a pentamer with 10 antigen-binding sites, the antibody has high avidity and could more effectively neutralize CHIKV antigen compared with bivalent IgG. Further neutralization studies of 3E7b and 8A2c on other cell lines can be performed to determine if there are cell type-specific factors involved in IgM-mediated CHIKV neutralization.

The efficacy of antibody neutralization is pre-determined by factors such as epitope affinity, accessibility and occupancy.^{41} The effective binding of 3E7b to CHIKV observed in IFA and indirect ELISA suggests that the mAb epitope(s) are situated conformationally on the surface of native CHIKV E1 or E2 protein. Because not all binding epitopes are neutralizing, we further identified a few amino acids that constitute or associate with the neutralizing epitope of 3E7b. Escape mutant CHIKV, namely, E24D, N218D and D233G were generated from 10 neutralization rounds under 3E7b selective pressure and they collectively localized to surface-exposed regions on CHIKV E2 ectodomain with high solvent accessibility. E24 is situated on the N-terminal beta-hairpin known as the N-flap, N218 is at the tip of domain C14/C15 and D233 is part of a 310 helix secondary structure.^{16} Of these 3 residues, N218 and D223 are spatially closest to each other in 13.0 Å, indicating that they could be within the footprint of an antibody paratope. Further analysis using infectious CHIKV mutant clone showed that only N218D mutation significantly reduced CHIKV binding (p < 0.0001) and resisted neutralization by 3E7b (Fig. 5), thereby highlighting that E2-N218 is a critical epitope for 3E7b binding and neutralization.

Based on these findings, we postulate a plausible mechanism of 3E7b-mediated CHIKV neutralization. The IgM binds to a
surface-exposed residue, N218, on viral E2 to form 3E7b-CHIKV aggregates that prevent virus attachment to the cell surface receptor. E2-N218 interaction with 3E7b might be associated with neighboring E24 and D223 residues within the antibody paratope where these residues indirectly stabilize 3E7b-CHIKV aggregates. Further Fab-docking studies are required to elucidate a detailed 3E7b binding mechanism to CHIKV E2 protein. As IgM binds with high flexibility to poliovirus in stellate and staple-like conformations, it may also be useful to unveil the binding conformation of 3E7b to CHIKV for better understanding on the mechanistic role of IgM in virus neutralization. Functionally, the role of E2-N218 in CHIKV neutralization has yet to be reported in other CHIKV-specific IgG studies. However, the residue might be an important antigenic determinant involved in alphavirus neutralization. Consistent with analysis on SINV escape variants that charged residues are important for antibody interaction, N218D mutation has resulted in substitution of a non-charged residue, Asn, to a charged Asp residue. In addition, a nearby residue, E2-N216 of RRV, SINV and Venezuelan equine encephalitis virus, was selected to confer virus escape from neutralizing antibodies. Mutation on E2-218 was also found to adapt RRV binding to heparan sulfate, the cell surface glycosaminoglycan involved in alphavirus receptor binding. Taken together, these studies suggest that N218 might be a conserved epitope associated in neutralization of other alphaviruses.

Understanding that CHIKV E1-A226V mutation and epistatic mutations on E2 protein have enabled CHIKV to adapt to a new *Aedes* mosquito vector, future mutation may occur on less conserved regions of the viral structural proteins. Hence,
neutralizing CHIKV in the serum. Due to its multivalency, IgM can efficiently mediate virus aggregation, thereby facilitating their recognition and destruction by cytotoxic T cells. However, the role of IgM in pre- and actual clinical setting is not well-understood. CHIKV-specific IgMs were found to persist in patients with chronic arthritis. Thus they could serve as biomarkers for CHIKV disease. In a broader context, analysis of IgM response in humans with alphavirus infection has revealed the potential usefulness of IgM as a diagnostic tool especially during endemics. Indeed, IgM-based ELISA has been developed for CHIKV serodiagnosis of adult patients infected with La Reunion strain. At present, there are no prior studies done on understanding the use of CHIKV-specific IgM as an immunotherapy in patients and animal models.

To evaluate in vivo efficacy of anti-CHIKV IgM, passive transfer of 3E7b was performed in our laboratory-established mouse model. Neonate mice are susceptible to CHIKV infection due to the lack of a fully-developed immune system. Hence, they can recapitulate hallmarks of CHIKV pathogenesis similar to clinical observations in patients. In this study, prophylactic dosage of 3E7b at 2 μg greatly improved disease outcome, with a survival of 80% and efficient virus clearance in the serum and CHIKV-targeted tissues, namely the limb, liver, spleen and brain. Relative to prior studies that achieved 80-100% survival with 4 μg or 250 μg of prophylactic anti-CHIKV mAbs, 2 μg of 3E7b is a potent dose against CHIKV infection. Recent studies showed that passive mAb transfer prior to CHIKV challenge protected mice against CHIKV-induced arthritis and swelling in the foot and joint. However, there are no studies evaluating mAb efficacy against CHIKV-induced myalgia. Moreover, because IgM has a short serum half-life and elicited response during the acute phase, we investigated if the protective efficacy of 3E7b could be sustained in the muscle tissues. We thus examined at day 7 p.i. the limb histology of neonate mice pre-treated with 20 μg of 3E7b. These mice showed healthy limb muscle morphology, in contrast to severe necrosis and inflammation in the control groups and other CHIKV mice models that revealed necrotizing myositis and mixed inflammatory infiltrates at day 7 p.i. This sustained protection against CHIKV-induced musculo-pathological changes implies that 20 μg of 3E7b is an optimal prophylactic dose.

To investigate whether 3E7b can be additionally protective in a post-infection setting, a single dose of 20 μg at 3E7b was administered to the mice at 4 h or 8 h after CHIKV challenge. Notably, 4 h post-treatment conferred 100% protection against CHIKV-induced lethality whereas protection was reduced to 50% when 3E7b was administered at 8 h p.i. As CHIKV caused high virus burden during the acute phase of infection, therapeutic dose of mAb is critical to improve the disease outcome. High virus load was detected in the limb muscles at day 2 p.i (Fig. 8C) and evident CHIKV-induced inflammation and necrosis was observed in some parts of the right limb muscle while other regions of the muscles retained healthy morphology at day 7 p.i (Fig. 9). This suggests that 3E7b could have partially protected against the onset of CHIKV-induced myalgia, possibly by supporting the host immunity and reducing CHIKV dissemination.

Figure 9. Histological analysis of limb muscles of mice post-treated with 3E7b after CHIKV infection. As described previously, limb muscles of infected mice at day 7 p.i. were harvested, fixed and processed for hematoxylin & eosin staining as described. Images are viewed and captured under 10× and 40× magnification of BX43 Olympus microscope. Representative images are shown with scale bar of 20 μm. Black arrowhead points to inflammatory cell. t, tendon.
to muscle tissues. Mice sera, liver and spleen showed that CHIKV was significantly cleared (p < 0.01) to levels below the limit of the detection assay (< 10 PFU/ml), while CHIKV clearance was less significant in the brain tissue (p < 0.05). Because liver, spleen and brain are more vascularized than the muscles, we postulate that 3E7b was delivered efficiently to neutralize or prevent CHIKV infection in these tissues.

In conclusion, we successfully generated and characterized 3E7b, a novel IgM mAb that potently neutralized CHIKV infection in cell-based and neonate murine models. As 3E7b protected mice from CHIKV disease, including CHIKV-induced myalgia, when given 24 h and 8 h prior to CHIKV infection, its potential merits in human patients were indicated. We also demonstrated that CH122508 and CH6708 belong to the East/Central/South African (ECSA) lineage, and they represent currently circulating CHIKV strains. CHIKV Ross (GenBank Accession number: FJ513654), named as CH6708, was used in mice experiments because this virus strain produced better clinical manifestations of CHIKV infection compared to CH122508 strain. These virus isolates were obtained from the serum of locally-infected patients and kindly provided by Environmental Health Institute of the National Environmental Agency (Singapore). Sequence analyses performed in our laboratory showed that CH122508 and CH6708 belong to the East/Central/South African (ECSA) lineage, and they represent currently circulating strains of CHIKV. CHIKV Ross (GenBank Accession number: AF490259), kindly provided by Dr. Ooi Eng Eong from DUKE-NUS, Sindbis virus (SINV) (GenBank Accession number: NC_001547) and Ross River Virus (RRV) (ATCC) were also used in the PRNT. To establish an infectious virus pool, confluent C6/36 cells were infected with local CHIKV strains, CHIKV Ross, RRV or SINV and maintained for 72 h to 96 h post-infection (p.i.).

Materials and Methods

Cells and virus culture

BHK-21 (ATCC No. CCL.10) cells were maintained in RPMI-1640 medium supplemented with 10% FCS and incubated at 37°C in 5% CO₂. C6/36 mosquito cells (ATCC No. CRL-1660) were maintained in L-15 medium with 10% heat-inactivated FCS and incubated at 28°C. Chikungunya virus SGEHICH122508 (GenBank Accession number: FJ445502) or named as CH122508 strain was used in all cellular experiments in this study. CHIKV LK(EH)/CH6708 (GenBank Accession number: FJ513654), named as CH6708, was used in mice experiments because this virus strain produced better clinical manifestations of CHIKV infection compared to CH122508 strain. These virus isolates were obtained from the serum of locally-infected patients and kindly provided by Environmental Health Institute of the National Environmental Agency (Singapore). Sequence analyses performed in our laboratory showed that CH122508 and CH6708 belong to the East/Central/South African (ECSA) lineage, and they represent currently circulating strains of CHIKV. CHIKV Ross (GenBank Accession number: AF490259), kindly provided by Dr. Ooi Eng Eong from DUKE-NUS, Sindbis virus (SINV) (GenBank Accession number: NC_001547) and Ross River Virus (RRV) (ATCC) were also used in the PRNT. To establish an infectious virus pool, confluent C6/36 cells were infected with local CHIKV strains, CHIKV Ross, RRV or SINV and maintained for 72 h to 96 h post-infection (p.i.).

Primary viral supernatant was collected and clarified by centrifugation at 290 g for 10 min before quantitating the virus titer by standard plaque assays. For use in indirect virion-based ELISA, a large pool of CH122508 strain was grown up by centrifugation at 25,000 rpm for 2.5 h at 4°C (Beckman L-90 and rotor SW41 Ti). Following which, virus pellet was resuspended in PBS and subjected to another round of ultracentrifugation. Purified CHIKV pellet obtained was then resuspended in PBS and plaque-assayed for virus titer.

Three-week old female BALB/c mouse (n = 5) were intraperitoneally injected with 800 µl of CH122508 strain (5 × 10⁷ PFU) for 3 times consecutively at 1-week interval. Mice serum was harvested after the second and third CHIKV challenge and analyzed via Western blotting for presence of immune response to CHIKV. After the fourth CHIKV boost, mouse spleen was harvested and splenocytes were fused with myeloma cells according to the instructions in ClonaCell-HY Hybridoma Cloning kit (StemCell Technologies, 03800). The resultant successful hybridoma cells were cultured according to a previously described protocol. Hybridoma clones were screened via IFA for the secretion of neutralizing anti-CHIKV mAbs in the crude culture supernatant. Positive hybridoma clones were further cloned by limiting dilution and expanded. Resultant subclones secreting neutralizing anti-CHIKV mAbs were further screened by immunofluorescence neutralization assay. Hybridoma subclones were then introduced into the peritoneal cavities of BALB/c mice. Following the production of ascites, mAbs were purified as described according to an optimized protocol and with the use of an IgM purification kit (Pierce, 44897). Final mAb purity and yield were analyzed by SDS-PAGE gel electrophoresis and BCA assay (Pierce, 23225), respectively. Purified mAb was isotyped with isostrip mouse monoclonal antibody isotyping kits (Roche, 11493027001).

Characterization of mAb antigenicity

(i) Immunofluorescence Assay

Confluent BHK cells on coverslips were infected with CHIKV CH122508 strain or single mutant CHIKV clones at MOI 10. At 24 h p.i. cells were fixed with methanol at −20°C, washed extensively with PBS and stained with mouse anti-CHIKV IgM, 3E7b or 8A2c at 1:100 dilution in PBS. Double-staining of mutant CHIKV-infected cells were performed using 3E7b and rabbit anti-E2 13893 B3 (in-house produced) at 1:100 or 1:300. Secondary antibody staining was performed with goat anti-mouse IgM FITC (Pierce, 31992) or anti-rabbit DyLight
594 conjugate (Pierce, 35560) at 1:300 or 1:500 dilution. Coverslips were washed with PBS after every staining step and finally mounted onto glass slide using Duolink in situ mounting medium with DAPI (Sigma-Aldrich, DUO82040). Images were captured at 10x and 100x under fluorescence microscopy of DAPI, FITC or TRITC channel (Olympus IX81 inverted microscope).

(ii) Indirect virion-based ELISA

Purified CHIKV was diluted in 50 μl of coating buffer (BD OptEIA, 550534) to a titer of 6 × 10^8 PFU and coated onto 96-well plate at 4°C overnight with shaking. Wells with coating buffer served as negative control. Following incubation, wells were washed with PBST buffer (PBS with 0.01% Tween-20) and BSA blocking buffer was added (2% BSA in PBST). After blocking at 37°C for 2h, wells were washed twice with PBST and anti-CHIKV IgM diluted to 0.1, 5, 10, 25, 50 or 100 ng, mouse mAb anti-CHIKV E2 8A4 IgG (a kind gift from Dr. Philippe Desprès, Pasteur Institute, France) or mouse IgM isotype clone GC323 (Millipore, MABC008) was added to each well. Concentration of CHIKV IgM was pre-quantitated by BCA assay. After the addition of primary mAbs, the ELISA plate was further incubated at 37°C for 1.5 h with shaking, washed thrice with PBST, and incubated with secondary anti-mouse IgM HRP at dilution of 1:5000 for another 1.5 h at 37°C with shaking. Wells were washed with a final round in PBST for 3 times and incubated with 100 μl of TMB substrate per well (BD OptEIA, 550534) for 10 min at room temperature. Reaction was stopped with 100 μl stop solution (BD OptEIA, 550534) and OD absorbance values were read at 450 nM using Tecan plate reader (Tecan i-control infinite 200). Negative controls consisting of “IgM + no CHIKV antigen coated” and blank wells consisting of “no IgM + no antigen coating” were included in the experiment. Mean OD values were expressed by subtracting negative control reading from positive “IgM + CHIKV coated wells” readings.

Neutralization Assay

Immunofluorescence neutralization assay screening

BHK-21 cells were seeded on 96-well plate to achieve 100% confluent monolayer. Equal volumes of CHIKV (CH122508 strain) of MOI 0.1 and culture supernatant of the hybridoma clone were mixed and incubated at 37°C for 1 h. Following that, the mixture was added to confluent BHK cells and CHIKV infection was carried out at 37°C for 1.5 h. Cells were washed with PBS twice and maintained in RPMI with 2% FCS. At 24 h p.i., cells were methanol-fixed and standard IFA was carried out as described above. Primary antibody staining was performed using mouse monoclonal anti-CHIKV E2 IgG (a kind gift from Dr. Philippe Desprès, Pasteur Institute of France) at 1:300 dilution for 1 h at 37°C, followed by goat anti-mouse IgG FITC (Pierce, 31992) at 1:500 dilution for the next 1 h. Cells were finally stained with DAPI and viewed under DAPI and FITC channel at 10x magnification (Olympus IX81 microscope).

Plaque Reduction Neutralization Test

PRNT assay was adapted and modified from reported studies. Mice ascites were complement-inactivated by heating at 55°C for 30 min prior to the assay. Anti-CHIKV IgM mAb at starting concentration of 0.25 μg/ml, 0.5 μg/ml or 1 μg/ml or mouse IgM isotype clone GC323 (Millipore, MABC008) at 1.7 μg/ml were 2-fold serially diluted in PBS. CHIKV (CH122508 strain) of 100 PFU was mixed with equal volume (1:1) of mAb for 1 h at 37°C. Following which, CHIKV-mAb mixture was added to confluent BHK-21 cell monolayer for 1.5 h at 37°C. Mock antibody control consists of CHIKV-PBS mixture. Following infection and PBS wash, cells were overlaid with 1% (w/v) carboxymethylcellulose (CMC/Aquacide II, Calbiochem, 9004-32-4) in RPMI supplemented with 2% FCS. After 72 h p.i., cells were stained with crystal violet dye containing 4% paraformaldehyde (PFA, Sigma-Aldrich, 158127). Relative percent of neutralization was calculated by expressing the total number of plaques per mAb dilution as a percentage of the number of plaques formed in PBS diluent (mock mAb control).

\[ \text{CHIKV Neutralization (\%)} = 100 - \left( \frac{\text{plaque number when incubated with mAb}}{\text{plaque number when incubated with PBS diluent}} \right) \times 100 \]

Values were fitted onto a dose-inhibition curve, from which non-linear regression was performed and IC50 was generated (Graphpad Prism 6). To validate cross-reactivity of 3E7b IgM with other strains of CHIKV or alphavirus, the above-described PRNT was also performed with CHIKV Ross strain as a representative of CHIKV East/Central/South African (ECSA) genotype, Ross River virus and Sindbis virus as representative of Semliki forest and Sindbis serocomplex, respectively. CHIKV Ross- and RRV-infected cells were crystal violet dye-fixed at 72 h p.i., while SINV-infected cells were fixed after 48 h p.i.

Post-binding neutralization assay

BHK-21 cells of 100% confluency were prechilled to 4°C before the cells were incubated with CHIKV 100 PFU for 1 h at 4°C. Cells were then gently washed twice with cold PBS, followed by incubation with mAb 3E7b or IgM isotype at 0.25 μg/ml diluted in PBS and RPMI with 2% FCS media for additional 30 min at 4°C. After which, cells were warmed to 37°C for 1 h to allow virus entry. Washes (3 times) with PBS were performed and infected cells were maintained in CMC overlay in RPMI media supplemented with 2% FCS. At 72 h p.i., cells were fixed with crystal violet dye and scored for plaques.

Neutralization escape mutant assay

CHIKV (CH122508 strain) was passaged consecutively under selective pressure from neutralizing 3E7b and under non-neutralizing condition using an equivalent mouse IgM isotype or PBS diluent. In brief, CHIKV of 10^3 PFU/ml was 10-fold serially diluted and each virus dilution was incubated at 1:1 volume with 3E7b 0.25 μg/ml, IgM isotype 0.5 μg/ml or PBS for 1 h at 37°C. Similar to PRNT, the mixture was used to infect confluent
BHK-21 cells that were later maintained in RPMI media with 3E7b or IgM isotype at respective concentration used for neutralization. Cells were monitored for a cytopathic effect (CPE) for the next 4 days and virus supernatant was then harvested from the wells with the highest dilution of virus that showed extensive CPE of estimated 80-100%. The supernatant was subjected to additional 9 rounds of passage in BHK cells as described above. At every 2 to 3 consecutive passage, viral supernatant was incubated with increasing concentration of 3E7b at 0.5 μg/ml, 1 μg/ml or 2.5 μg/ml, respectively. After the 10th passage, the harvested viral supernatant was plaque-purified in BHK cells. In brief, cells infected with the viral supernatant were overlaid with 0.5% agarose in RPMI media containing 3E7b 2.5 μg/ml, IgM isotype 5 μg/ml or PBS. Following 48 h to 72 h p.i., visible plaques were isolated individually and further propagated in BHK cells for one passage. Viral supernatant was harvested at 48 h p.i., when extensive CPE occur, and this was subjected to another round of plaque purification as described above.

Viral RNA extraction, reverse-transcription and sequencing

CHIKV genomic RNA was extracted from the viral supernatant of escape clones using QIAamp viral RNA mini kit (Qiagen, 52906) according to the manufacturer’s protocol. Eluted CHIKV RNA was reversed transcribed by MMLV Reverse transcriptase (Promega, M7122) and primers designed (Sigma-Aldrich) to flank the complete CHIKV E1 or E2 gene. PCR products were electrophoresed, gel extracted and spin-column purified according to the manufacturer’s protocol (Qiagen, 28106). Purified PCR products were sequenced at CHIKV E1 or E2 gene and resulting sequences were analyzed using SeqTrace software.63

Computational analyses of neutralization epitopes

CHIKV E1/E2 protein structure of CH122508 strain was predicted based on the published CHIKV mature glycoprotein complex (PDB code: 3N43) by molecular superimposition using Phyre 2 software (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id-index).64 Visualization and distance quantification of single epitopes were performed on Pymol software (www.pymol.org).65 Single-point mutation was highlighted in the respective colors. Calculation of surface accessibility was additionally performed using POPS algorithm (http://mathbio.nimr.mrc.ac.uk/wiki/POPS).68

CHIKV mutant clone generation

Site-directed mutagenesis inverse PCR

Site-directed mutagenesis of CHIKV E2 gene was carried out by inverse PCR cycling using CH122508 full-length genomic DNA pSMART-LCKan vector (Lucigen, 40821-2; GenBank Accession number: AF532106) that was established previously in our laboratory. Three sets of PCR primers (Sigma-Aldrich) were constructed where each pair of forward and reverse primer contains the desired single-base escape mutation in E2 (E24D, N218D and D223G). Inverse PCR reaction was carried out using Long PCR enzyme mix (ThermoScientific, K0181) following manufacturer’s conditions. PCR products were digested by Dpn-I (NEB, R0176S) at 37°C for 6 h and transformed into competent Escherichia coli strain XL10 gold in 1:5 ratio. Transformed culture was spread onto LB agar supplemented with 30 μg/ml kanamycin (MP Biomedicals, 0219453105) and incubated overnight at 30°C. Plasmid from colonies were isolated by Pureyeld miniprep kit (Promega, A1222) and subjected to digestion by BglII (NEB, R0144S) and Smal (NEB, R0141S) at 37°C for 1 h. After visualization on 0.7% ethidium bromide gel, positive clones were DNA sequenced using CHIKV E2 forward primer to confirm the presence of desired mutation (1st base sequencing, Singapore). Successful bacterial clones were further expanded, plasmid-extracted and column-purified.

In vitro transcription (IVT) of full-length virus mutant clone

CHIKV mutant DNA plasmid clones of 4 μg were linearized with NotI enzyme (NEB, R0189S) at 37°C overnight. Complete linearization was verified by gel electrophoresis and 1 μg of the linearized plasmid was subjected to IVT reaction using mMESSAGE mMACHINE T7 transcription kit (Life Technologies, AM1344). After 2 h incubation at 37°C, the transcribed RNA was recovered using RNeasy Mini kit (Qiagen, 74106) and quantitated at 260 nm by NanoDrop 2000 spectrophotometer (ThermoScientific).

Viral RNA Transfection

Confluent BHK-21 cells in 24-well plates were transfected with 1 μg of CHIKV RNA-Lipofectamine 2000 complex (Life Technologies, 11668-019) and maintained in RPMI supplemented with 2% FCS. Cells were observed for presence of CPE in the 48 h to 72 h p.i. Harvested supernatant was clarified and further passaged once in C6/36 cells to establish an infectious CHIKV pool. DNA sequencing of entire length of CHIKV mutant cDNA clone was performed using a set of optimized CHIKV-specific primers established previously.

MAb immunotherapy in mice model

For prophylaxis treatment, 5-day old neonate mice (n = 5 or 6 per group) were pre-treated with 0.2 μg, 2 μg, 20 μg or 200 μg of anti-CHIKV 3E7b or a purified mouse IgM isotype (Bethyl laboratories, M110-100) diluted in sterile PBS or mock-treated with PBS by intraperitoneal (i.p) injection at 24 h and 8 h prior to CHIKV infection. Following this, mice were i.p injected with 50 μl of CHIKV at 4 x 10^5 PFU (strain LK(EH) CH670, GenBank Accession number: FJ513654). For therapeutic study, 6-day old neonate mice were given CHIKV by i.p injection, followed by administration of 3E7b, IgM isotype or PBS at 8 h or 4 h p.i. To investigate virus clearance in the tissues and serum, mice were sacrificed at 48 h p.i. by CO2 overdose and whole blood, brain, spleen, liver and muscle of the hind limbs were harvested for quantification of CHIKV titer by virus plaque assay. Tissues were suspended in 1 ml PBS and homogenized using Precellys CK28 beads (Bertin Technologies, KT03961-1-002.2). Blood and homogenized tissues were then
Here, noted and all mice were euthanized by CO2 overdose and fixed in 4% PFA for 3 days at room temperature. Hind limbs were decalcified in Surgipath decalcifying solution II (Leica, DC3800460II) for an additional 2 h. Following which, tissues were stepwise dehydrated in 50% and 70% ethanol, paraffin-embedded and sectioned to 4 μm before staining with Hematoxylin and Eosin (H&E). Images were captured using BX43 Olympus microscope at ×10 and ×40 magnification.

Statistical analysis

In accordance with our approved IACUC protocol guidelines, a minimum number of mice were used in this study without compromising the statistical power of our findings. For survival analysis, sample size (n) required in a time to event occurrence is calculated using the following equation.66

\[
n = C \frac{p \cdot q \cdot n}{d^2} + 2 + \frac{2}{d} + 2
\]

Here, \( p \) is defined as the estimated proportion of mice in the control group that will succumb to CHIKV infection. \( q \) is the desired proportion of mice in the treatment group that will succumb to CHIKV infection, \( d \) is given as \( p–q \), and \( C \) is 10.51 given a significance level of 0.05 and confidence level of 90%. As for CHIKV load quantification, sample size is calculated using the following equation.66

\[
n = 1 + 2C \left( \frac{s^2}{d^2} \right)
\]

Here, \( s \) is defined as the group standard deviation and \( d \) as the desired treatment effect. \( C \) is 10.51 given a significance level of 0.05 and confidence level of 90%. Using these equations, \( n = 5 \) or 6 mice used in our study is determined to have sufficient statistical power to detect at a significant level of 0.05, if there is any different effect between PBS (mock mAb) and mAb treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher’s website.

References

1. Fournier ED, Morrison JG. Rheumatoid arthritis syndrome after chikungunya fever. S Afr Med J 1979; 56:130-2; PMID:694934.
2. Brighton SW, Prosecky OW, de la Harpe AL. Chikungunya virus infection. A retrospective study of 107 cases. S Afr Med J 1983; 63:313-5; PMID:6298956.
3. Kee AC, Yang S, Hamnett-Tamblyn P. Artyclochikungunya virus infections in immunocompromised patients. Emerg Infect Dis 2010; 16:1038-40; PMID:20507772; http://dx.doi.org/10.3200/eid1606.091115.
4. Hoarau J, Jaffar Bandjee MC, Kriebich Trontot P, Das T, Li-Pat-Yuen G, Dassa B, Denizor M, Guichard E, Ribera A, Henri T, et al. Persistent chronic inflammation and infection by Chikungunya arbovirus in spite of a robust host immune response. J Immunol 2010; 184:5914-27; PMID:20404278; http://dx.doi.org/10.4049/jimmunol.0900251.
5. Pellet AS, Alessandri JL, Robin S, Samperio S, Attal T, Brayer C, Pasquet M, Jaffar-Bandjee MC, Benhamou LS, Tiran-Rajaofera I, et al. Severe forms of chikungunya virus infection in a pediatric intensive care unit on Reunion Island. Med Trop (Mars) 2012; 72 Spec No:88-93; PMID:22693937.
6. Robin S, Ramful D, Le Seach F, Jaffar-Bandjee MC, Rigou G, Alessandri JL. Neurologic manifestations of pediatric chikungunya infection. J Child Neurol 2008; 23:1028-35; PMID:18287573; http://dx.doi.org/10.1177/0883078008314151.
7. Schuffenecker I, Iremam I, Michaut A, Murri S, Frangeul L, Vaney MC, Lavrent R, Pardigon N, Reynes JM, Pertinelli F, et al. Genome microevolution of chikungunya viruses causing the Indian Ocean outbreak. PLoS Med 2006; 3:e263; PMID:16700631; http://dx.doi.org/10.1371/journal.pmed.0030263.
8. Njenga MK, Ndirangu J, Logue CH, Kelly GH, Sang R, Sergon K, Beimann R, Powers AM. Tracking epidemic Chikungunya virus into the Indian Ocean from East Africa. J Gen Virol 2008; 89:2754-60; PMID:18931072; http://dx.doi.org/10.1177/0883073808314151.
9. Njenga MK, Ndirangu J, Logue CH, Kelly GH, Sang R, Sergon K, Beimann R, Powers AM. Tracking epidemic Chikungunya virus into the Indian Ocean from East Africa. J Gen Virol 2008; 89:2754-60; PMID:18931072; http://dx.doi.org/10.1177/0883073808314151.
10. Pullmanasahakul R, Roiyakul S, Aevaratukul P, Smith DR. Chikungunya in Southeast Asia: understanding the emergence and finding solutions. Int J Infect Dis 2011; 15:e671-6; PMID:21775183; http://dx.doi.org/10.1016/j.ijid.2011.06.002.
11. Viennet E, Knope K, Faddy HM, Williams CR, Harley D. Assessing the threat of chikungunya virus emergence in Australia. Commun Dis Intell Q Rep 2013; 37: E136-43; PMID:24168087.
12. Dogan AD, Bunes K, Skarpheidsson S. The tropical disease Chikungunya fever has come to Europe. Ugeskr Laeger 2013; 175:176-9; PMID:23763933.
13. Powers AM. Risks to the Americas associated with the continued expansion of chikungunya virus. J Gen Virol 2015; 96:1-5; PMID:25239764; http://dx.doi.org/10.1177/0022131715616682.
14. Van Boeckel TP, Dobson F, Rosine J, Blateau A, Rousset D, Mathews S, Leparc-Goffart I, Fluin O, Pratt C, Cesaire R, et al. Chikungunya outbreak in the Caribbean region, December 2013 to March 2014, and the significance for Europe. Euro Surveill 2014; 19: pii=207579.
15. Strauss JH, Strauss EG. The alphaviruses: gene expression, replication, and evolution. Microbiol Rev 1994; 58:491-562; PMID:7968923.
16. Voss JE, Vaney MC, Duquesney S, Vorvoreanu C, Girard-Blanc C, Cribler E, Thompson A, Bricogne G, Rey FA. Glycoprotein organization of Chikungunya virus particles revealed by X-ray crystallography. Nature 2010; 468:709-12; PMID:21124458; http://dx.doi.org/10.1038/nature09555.
17. Mukhopadhyay S, Zhang W, Gabler S, Chipman PR, Strauss EG, Strauss JH, Baker TS, Kuhn RJ, Rossmann MG. Mapping the structure and function of the E1 and E2 glycoproteins in alphaviruses. Structure 2006; 14:63-73; PMID:16407666; http://dx.doi.org/10.1016/j.str.2005.07.025.
18. Smith TJ, Cheng RH, Olson NH, Peterson P, Chase E, Kuhn RJ, Baker TS. Putative receptor binding sites on alphavirus as visualized by cryoelectron microscopy. PNAS 1995; 92:10648-52; PMID:7479858; http://dx.doi.org/10.1073/pnas.92.23.10648.

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62. Lok SM, Kostyuchenko V, Nybakken GE, Holdaway HA, Battisti AJ, Sukupolvi-Petty S, Sedlak D, Fremont DH, Chipman PR, Roehrig JT, et al. Binding of a neutralizing antibody to dengue virus alters the arrangement of surface glycoproteins. Nat Struct Mol Biol 2008; 15:312-7; PMID:18264114; http://dx.doi.org/10.1038/nsmb.1382

63. Stucky B. SeqTrace: a graphical tool for rapidly processing DNA sequencing chromatograms. J Biomol Tech 2012; 23:90-3; PMID:22942788; http://dx.doi.org/10.7171/jbt.12-2303-004

64. Kelley L, Sternberg M. Protein structure prediction on the web: a case study using the Phyre server. Nat Protoc 2009; 4:363-71; PMID:19247286; http://dx.doi.org/10.1038/nprot.2009.2

65. Schrodinger L. The PyMOL molecular graphics system, version 1.3 r1. New York: Oxford University Press; 2010.

66. Dell RB, Holleran S, Ramakrishnan R. Sample Size Determination. ILAR J 2002; 43:207-13; PMID:12391396; http://dx.doi.org/10.1093/ilar.43.4.207

67. Brihis AC, Rubrechtb L, Navarro-Sancheza ME, Marichala V, Frenkiela MP, Lapaludb P, Launeb D, Sallc AA, Despies P. Production and characterization of mouse monoclonal antibodies reactive to Chikungunya envelope E2 glycoprotein. Virol J 2008; 371:185-95; http://dx.doi.org/10.1016/j.virol.2007.09.028