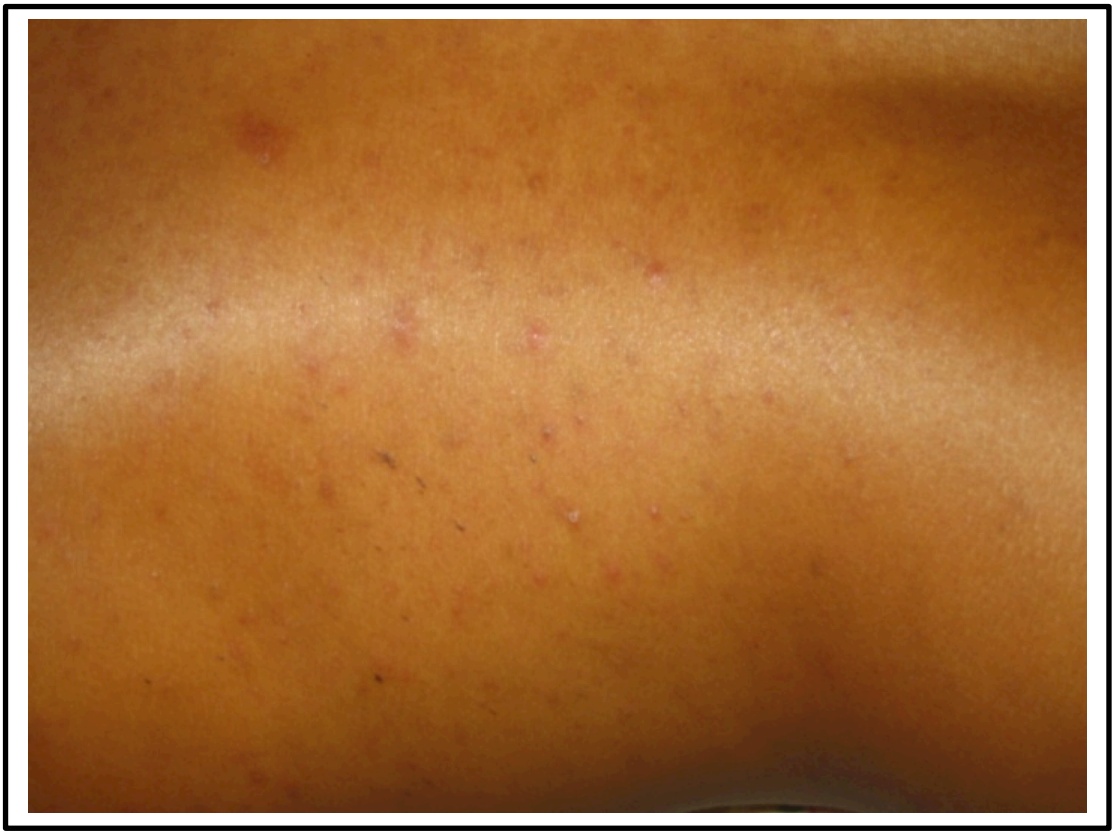

Figure S1. Papular rash at time of acute presentation. The subject presented to the primary care physician with a fever (102°F) of three days duration, with concurrent development of bilateral joint pain in elbows and fingers, and rash. Providers noted a raised, non-pruritic, blanching, papular rash (photograph shown in the figure) across the back, chest and abdomen.

Figure S2. Smith *et al.*

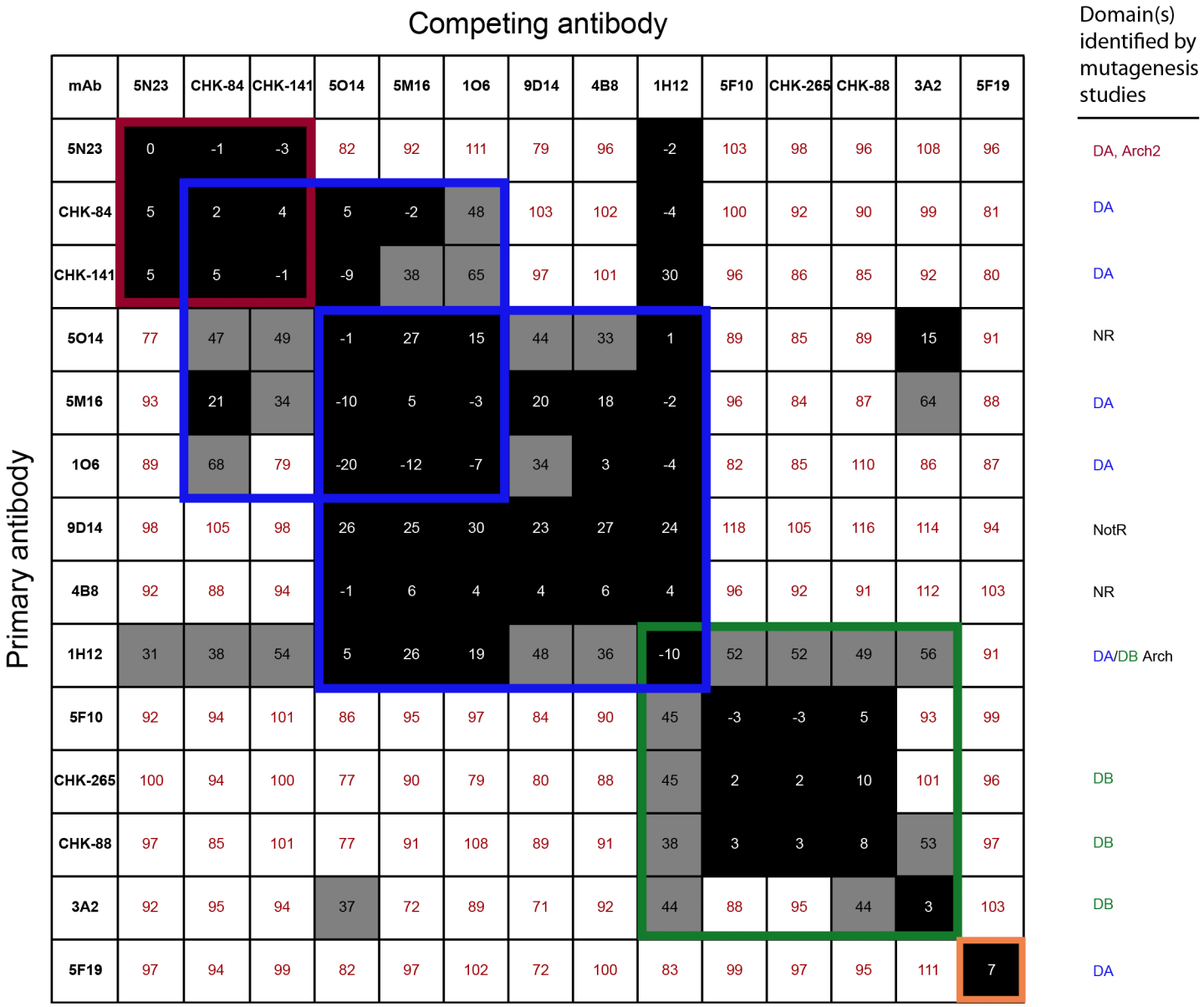

Figure S2. Identification of mAb competition groups. Quantitative competition binding using Octet-based biolayer interferometry was used to assign mAbs to competition groups. Anti-Penta-His biosensor tips covered with immobilized CHIKV-LR2006 E2 ectodomain were immersed into wells containing primary mAb, followed by immersion into wells containing competing mAbs. The values shown are the percent binding of the competing mAb in the presence of the first mAb (determined by comparing the maximal signal of competing mAb applied after the first mAb complex to the maximal signal of competing mAb alone). MAbs were judged to compete well for binding to the same site if maximum binding of the competing mAb was reduced to <30% of its non-competed binding (black squares) or to exhibit partial completion if the binding of the competing mAb was reduced to < 70% of its non-competed binding (gray squares). MAbs were considered non-competing if maximum binding of the competing mAb was > 70% of its non-competed binding (white squares). Four competition-binding groups were identified, indicated by colored boxes (group 1 in red, group 2 in blue, group 3 in green, and group 4 in orange). The corresponding major antigenic sites for mAbs discovered by alanine-scanning mutagenesis (Table 1 and Figure 1) are summarized in the columns to the right of the competition matrix. DA indicates domain A; DB indicates domain B, e indicates both arch 1 and 2; NT indicates not tested; NotReact indicates that the mAb did not react against the wild-type envelope proteins; NoReduct indicates the mAb did bind to the wild-type E proteins, but no reduction was noted reproducibly for any mutant. The data are combined from one experiment, with multiple readings for each mAb alone and a single reading of a mAb in combination with each competing antibody.

Figure S3. Smith *et al.*

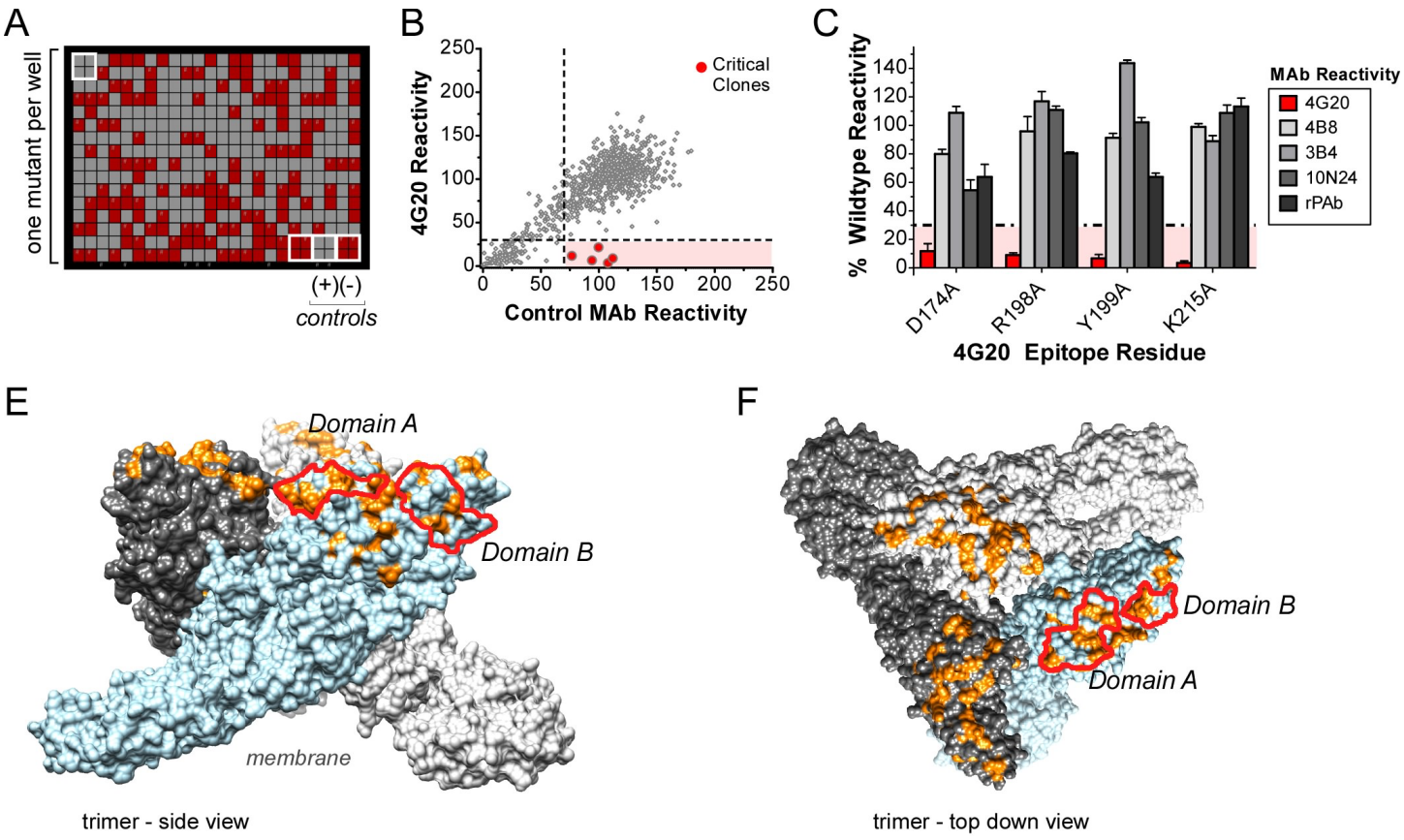

Figure S3. High resolution epitope mapping of CHIKV MAbs. (A) An alanine scanning mutation library for CHIKV envelope protein encompassing 910 E2/E1 mutations was constructed where each amino acid was individually mutated to alanine. Each well of each mutation array plate contains one mutant with a defined substitution. A representative 384-well plate of reactivity results is shown. Eight positive (wild-type E2/E1) and eight negative (mock-transfected) control wells are included on each plate. **(B)** For epitope mapping, human HEK-293T cells expressing the CHIKV envelope mutation library were tested for immunoreactivity with a MAb of interest (MAb 4G20 shown here) and measured using an Intellicyt high-throughput flow cytometer. Clones with reactivity <30% relative to wild-type CHIKV E2/E1 yet >70% reactivity for a different CHIKV E2/E1 MAb were initially identified as critical for MAb binding. **(C)** Mutation of four individual residues reduced 4G20 binding (red bars) but did not greatly affect binding of other conformation-dependent MAbs (gray bars) or rabbit polyclonal antibody (rPAb, a gift from IBT Bioservices). Bars represent the mean and range of at least two replicate data points. **(D)** The epitopes of neutralizing MAbs with $PRNT_{50}$ < 1,000 ng/ml are mapped onto the trimeric crystal structures of E2/E1 (PDB Entry 2XFC). All neutralizing epitopes map to well-exposed, membrane-distal domains of E2/E1. Each individual E2/E1 heterodimeric subunit is shown in a different color for clarity. Highly immunogenic regions in E2 domains A and B which contain critical epitope residues for multiple MAbs are outlined in red on a single subunit of E2.

Figure S4. Smith *et al.*

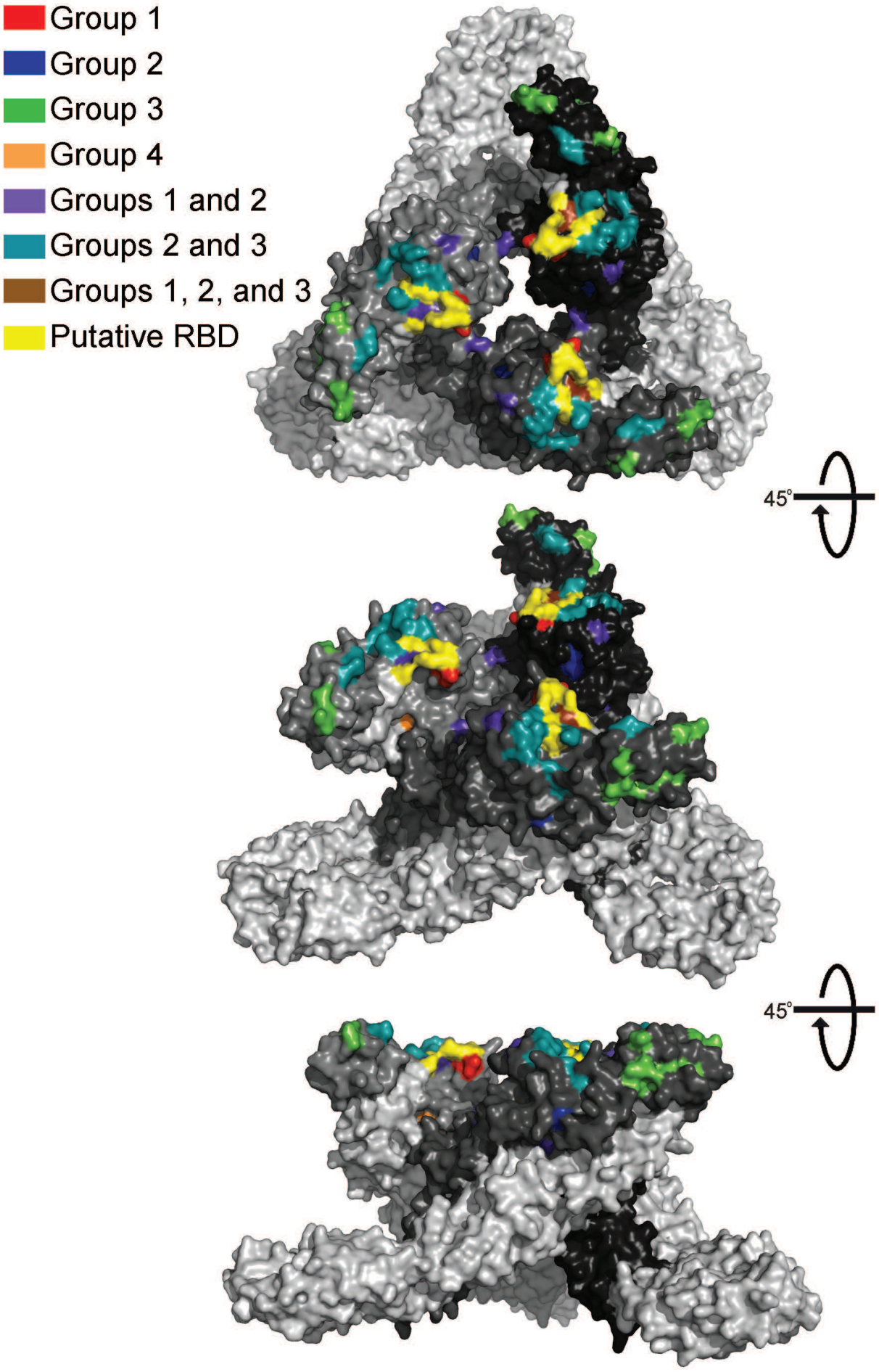

Figure S4. Structural analysis of E2 residues important for mAb binding for antibodies mapped to competition groups. Location of residues required for binding of the human or mouse mAbs from different competition groups (Fig. 1) mapped onto the crystal structure of E1/E2 (PDB ID 2XFB). A space-filling model of the E1/E2 trimer with E1 colored in white and each E2 monomer colored with light grey, dark grey, or **black**. The residues required for antibody binding are color-coded according to the competition group(s) to which they belong.

Red indicates residues D117 and I121, which are required for binding of 5N23, and belong to competition group 1.
Blue indicates residues R80 and G253, which are required for binding by I06 or 5M16, belong to competition group 2.
Green indicates residues Q184, S185, I190, V197, R198, Y199, G209, L210, T212, and I217, which are required for binding by CHK-285, CHK-88, or 3A2, and belong to competition group 3.
Orange indicates residues H18, which is required for binding of 5F19, and belongs to competition group 4.
Purple indicates residues E24, A33, L34, R36, V50, D63, F100, T155, which are required for binding by 5N23, CHK-84, or CHK-141, and belong to competition groups 1 and 2.
Teal indicates residues T58, D59, D60, R68, I74, D77, T191, N193, and K234, which are required for binding by 1H12, and belong to competition groups 2 and 3.
Brown indicates residues D71, which is required for binding by CHK-84 and 1H12, and belongs to competition groups 1, 2, and 3.
Yellow indicates residues (T58, D71, N72, I74, P75, A76, D77, S118, and R119) that comprise the putative receptor-binding domain (RBD), with the exception of residue D71, which belongs to competition groups 1, 2, and 3.

The **upper panel** shows a bird's eye view of the trimer, the **middle panel** shows an angled side view of the trimer rotated 45 in the x-axis from the structure in the upper panel, and the **bottom panel** shows a side view of the trimer rotated 45 in the x-axis from the structure in the middle panel.

Table S1. Kinetics of human CHIKV antibodies binding antigen measured by SPR

Values for ka, kd, *K*D are means ± standard deviations. *K*D = kd/ka; $t_{1/2}$ = (ln(2)/kd)/60.

CHKV MAb	Ligand	ka (10^5M-1s-1)	kd (10^{-4}s-1)	*K*D (nM)	$t_{1/2}$ (min)
5M16	p62-E1	1.09 ± 0.02	1.13 ± 0.02	1.03 ± 0.01	102 ± 2
5M16 Fab	VLP	1.19 ± 0.01	0.84 ± 0.13	7.07 ± 1.06	137 ± 21
4J21	p62-E1	1.19 ± 0.02	0.62 ± 0.04	0.54 ± 0.31	186 ± 11
4J21 Fab	VLP	1.58 ± 0.03	14.2 ± 0.29	9.00 ± 0.03	8 ± 0.2
3E23	p62-E1	3.18 ± 2.43	2.67 ± 0.87	6.11 ± 1.41	43 ± 19
3E23 Fab	VLP	0.203 ± 0.03	3.93 ± 0.40	19.6 ± 3.16	29 ± 3
4B8	p62-E1	2.98 ± 2.98	5.06 ± 1.10	5.57 ± 1.10	23 ± 5
4B8 Fab	VLP	0.60 ± 0.04	3.33 ± 0.30	5.60 ± 0.46	35 ± 3
5N23	p62-E1	2.98 ± 0.75	2.40 ± 0.66	0.87 ± 0.37	48 ± 17
1L1	p62-E1	0.88 ± 0.44	2.73 ± 0.36	3.79 ± 2.10	42 ± 6
2C2	p62-E1	1.54 ± 0.65	6.08 ± 1.45	4.59 ± 2.32	19 ± 6
2D12	p62-E1	17.5 ± 1.64	5.41 ± 4.97	0.49 ± 0.08	13 ± 4
4N12	p62-E1	1.24 ± 0.02	1.18 ± 0.01	0.95 ± 0.02	98 ± 1
5O14	p62-E1	0.79 ± 0.02	9.11 ± 0.19	1.15 ± 0.01	13 ± 0.3
9D14	p62-E1	2.70 ± 0.90	2.82 ± 0.14	1.13 ± 0.40	41 ± 2
8G18	p62-E1	3.90 ± 0.21	1.88 ± 0.11	0.48 ± 0.02	62 ± 3
4J14	p62-E1	1.61 ± 0.47	15.3 ± 2.52	9.94 ± 2.64	8 ± 1
5F	p62-E1	2.61 ± 0.04	50.9 ± 0.75	19.5 ± 0.2	2 ± 0.03
3A2	p62-E1	2.12 ± 0.02	10.1 ± 0.12	4.78 ± 0.08	11 ± 0.1
1M9	p62-E1	1.86 ± 0.99	3.98 ± 0.26	2.48 ± 0.99	29 ± 2
3B4	p62-E1	2.91 ± 0.09	1.56 ± 0.11	0.54 ± 0.02	74 ± 5
8B	p62-E1	0.99 ± 0.03	2.74 ± 0.10	2.77 ± 0.18	42 ± 2
8E22	p62-E1	0.48 ± 0.02	2.0 ± 0.13	4.22 ± 0.42	58 ± 4

Supplemental Experimental Procedures

Human subject and peripheral blood cell isolation

An otherwise healthy adult subject presented in October of 2006 with CHIKV infection. The symptoms of CHIKV infection coincided with return from a one-year visit to Sri Lanka, during which the patient spent time in urban areas (primarily Colombo), and rural settings, including rainforests and coastal areas. The patient experienced multiple insect bites over the course of the visit, but remained in good health throughout the stay. On return to the U.S., the subject presented to the primary care physician with a fever (102°F) of three days duration. The patient reported the concurrent development of bilateral joint pain in elbows and fingers, and a raised, non-pruritic rash on the back and abdomen, accompanied by general "body ache" and headache. On presentation, he appeared to be well, and in no acute distress. A mild, blanching, papular rash extended across the back, chest and abdomen (see **Figure S1**). A mild conjunctivitis was noted. The skeletal exam was remarkable for tender swollen fingers, knees and elbows, which were without erythema or effusions. Muscle strength and range of motion of the affected joints were intact, but joint movement elicited pain.

Blood was drawn for a CBC, serologies and malaria smears, and the patient was discharged. The white blood cell count was 4.0×10^4 cells/mm^3, the hematocrit was 41% and platelet count was 180,000/mm^3. The total lymphocyte count was 1.0×10^4 cells/mm^3. Malaria smears and serologies were negative, and the patient was diagnosed tentatively as having a viral illness of unknown etiology.

The patient returned to the clinic two weeks later, afebrile, but with persistent arthralgia, most prominent in the fingers. The patient described the pain and stiffness as no better, and perhaps worse, than during his previous visit. The patient reported that an outbreak of chikungunya was occurring in the area of previous travel. Blood was drawn and serum separated and sent to CDC for PCR and serological testing, which confirmed the diagnosis of chikungunya infection.

In April 2012, five and a half years after the index infection, peripheral blood mononuclear cells (PBMCs) were isolated by density gradient separation on Ficoll without known exposure to CHIKV or other arthritogenic alphaviruses in the intervening period while living in the United States. The cells were cryopreserved and stored in liquid nitrogen until study. The protocol for recruiting and collecting blood samples from subjects was approved by the Institutional Review Boards of the University of North Carolina at Chapel Hill and the Vanderbilt University Medical Center.

Generation of human hybridomas

Cryopreserved PBMC samples were thawed rapidly at 37ºC and washed prior to transformation with Epstein-Barr virus, as described (*1*). Cultures were incubated at 37ºC with 5% CO_2 for 10 days and screened for the presence of cells secreting CHIKV-specific antibodies in the supernatant using VRP neutralizing assays and an ELISA. We performed two independent transformations using separate aliquots of the same blood sample.

In the first transformation, we established 3,840 cultures (10 x 384-well plates) containing an average of 42 transformed B cell colonies per culture, for an estimated total of about 161,000 individual B cell colonies. To screen for antibodies that display neutralizing activity against CHIKV under BSL2 conditions, we developed a high-throughput fluorescence reduction neutralization assay using CHIKV replicon particles (VRPs) that express green fluorescent protein as a reporter. VRPs are virions that display the native viral glycoproteins but lack the full-length viral genome and thus are incapable of generating infectious progeny (*2*). We used VRPs derived from strain SL15649 (*3*), which was isolated from Sri Lanka in 2006. SL15649 is contemporaneous to the strain that infected our donor and is likely very similar in sequence. From this experiment, we identified 160 B cell cultures with supernatants that mediated neutralization at 90% inhibition, suggesting a frequency of 0.099% virus-specific B cells per total B cells (~ 1 in 1,000). A total of 60 of these lines inhibited at a level of > 98%, and in the secondary screen, supernatants from 58 of the 60 lines contained antibodies that bound in

ELISA to cell-culture-produced CHIKV (strain 181/25) captured on an immunoassay plate. We selected 35 of the 58 lines with the highest neutralizing and binding activity for hybridoma fusion, identified 22 hybridomas with virus-binding supernatants after fusion and plating, and successfully isolated 14 clones for further study. In the second transformation, we established 1,536 cultures (4 x 384-well plates) containing an average of 38 transformed B cell colonies per culture, for an estimated total of about 58,000 individual B cell colonies tested, suggesting a virus-specific B cell frequency of 0.1% (again, ~ 1 in 1,000). In this experiment, we used a primary screen of ELISA binding to CHIKV strain 181/25 without a prior neutralizing test. We identified 60 lines with ELISA optical density signal greater than four times the background level, selected the 30 B cell lines with the highest optical density signal in ELISA for fusion, identified 18 hybridomas with virus-binding supernatants after fusion and plating, and successfully isolated 16 clones for further study.

Fusion with myeloma cells. Cells from wells with supernatants capable of neutralizing CHIKV infectivity were fused with HMMA2.5 non-secreting myeloma cells as described (*1*). Resultant hybridomas were selected by growth in hypoxanthine-aminopterin-thymidine (HAT) medium containing ouabain, biologically cloned by single-cell FACS using a FACSAria III cell sorter (BD Biosciences), and expanded.

Human mAb production and purification

Wells containing hybridomas producing CHIKV-specific antibodies were cloned by three rounds of limiting dilution or with a ClonePix device (Molecular Devices) according to the manufacturer's instructions. Once individual clones were obtained, each hybridoma was expanded until 50% confluent in 75 cm^2 flasks. For antibody expression, cells were collected with a cell scraper, washed in serum-free medium (GIBCO Hybridoma-SFM from Invitrogen, 12045084), and divided equally into four 225 cm^2 flasks (Corning, 431082) containing 250 mL serum-free medium. Cells were incubated for 21 days before medium was clarified by

centrifugation and passed through a 0.2 μm sterile filter. Antibodies were purified from clarified medium by protein G chromatography (GE Life Sciences, Protein G HP Columns).

Cells

BHK-21 cells (ATCC CCL-10) were maintained in alpha minimal essential medium (αMEM; Gibco) supplemented to contain 10% fetal bovine serum (FBS) and 10% tryptose phosphate (Sigma). Vero 81 cells (ATCC CCL-81) were maintained in αMEM supplemented to contain 5% FBS. Medium for all cells was supplemented to contain 0.29 mg/mL L-glutamine (Gibco), 100 U/mL penicillin (Gibco), 100 μg/mL streptomycin (Gibco), and 500 ng/mL amphotericin B. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2.

Viruses used in this study

CHIKV East/Central/South African [ECSA] genotype strains used in this study include LR2006_OPY1 [accession number DQ443544.2; provided by Stephen Higgs (Manhattan, KS)], S27 (accession number AF369024.2), and SL15649 (accession number GU189061). The West Afrrican genotype strain used in this study is NI 64 IbH 35 (accession number HM045786.1). CHIKV Asian genotype strains used in this study include vaccine strain 181/25 (accession number L37661.3), Caribbean strain 99659 [accession number KJ451624; isolated in 2014 from a subject in the British Virgin Islands (*11*)], and RSU1 (accession number HM045797.1). Strains IbH35, RSU1, and 99659 were provided by Robert Tesh (World Reference Center for Emerging Viruses and Arboviruses, Galveston, TX).

Generation of CHIKV VRP plasmid constructs

A three-plasmid CHIKV replicon helper system was derived from a plasmid containing the full-length cDNA of the CHIKV strain SL15649 genome sequence using PCR-based cloning methodologies. A CHIKV replicon

genome was constructed using a two-step process that involved the generation of an intermediate cloning vector with the CHIKV full-length structural cassette substituted with a multiple cloning site (MCS). Enhanced green fluorescent protein (eGFP) was subcloned into the multiple cloning site of this plasmid to generate pMH41 (CHIKV SL15649 eGFP replicon). The construction of a two-plasmid helper system included a multi-step cloning process that first involved the generation of a full-length structural gene helper plasmid via removal of the majority (6,891 nt) of the CHIKV non-structural cassette. The full-length structural cassette was further subdivided into two constructs, pMH38 (CHIKV SL15649 capsid helper), which is comprised of the capsid gene sequence followed by a unique AvrII restriction site, and pMH39 (CHIKV SL15649 glycoprotein helper), which contains an in-frame deletion of the capsid RNA-binding domain followed by the intact envelope glycoprotein (E3-E1) coding sequence.

Recombinant CHIKV p62-E1 production

A plasmid containing CHIKV p62 (*i.e.*, E3 [aa S1-R64] - E2 [aa S1–E361] -16 amino acid linker - E1 [aa Y1-Q411] followed by a His tag) (*4*) was transfected into 293F cells using 293fectin reagent (Invitrogen). After 72 hours incubation, the supernatant was removed, and the cells were cultured for an additional 72 hours. The pooled supernatants were loaded onto a nickel agarose bead column (GoldBio) and eluted with imidazole. The protein was further purified using a Superdex S200 gel filtration column (GE Life Sciences). Fractions containing the CHIKV p62-E1 protein were pooled, frozen, and stored at -80°C.

Generation of CHIKV strain SL15649-derived VRP stocks

VRP stocks were recovered from recombinant CHIKV plasmids in a certified biological safety level 3 (BSL3) facility in biological safety cabinets in accordance with protocols approved by the Vanderbilt University Department of Environment, Health, and Safety and the Vanderbilt Institutional Biosafety Committee. The

three SL15649 replicon system plasmids were linearized by digestion with NotI-HF, purified by phenol-chloroform extraction, and used as templates in transcription reactions using an mMessage mMachine SP6 transcription kit (Life Technologies) to produce capped, full-length RNA transcripts *in vitro*. Viral RNA transcripts were introduced into BHK21 cells by electroporation using a GenePulser electroporator. Culture supernatants containing VRPs were collected 24 hours after electroporation; supernatants were clarified by centrifugation at 855 × g for 20 min, aliquoted, and stored at -80 °C. VRP stocks were evaluated for propagation-competent recombinant virus by serial passage of 20% of the stock and 10% of passage 1 culture supernatant using Vero81 cells, which were examined for cytopathic effect (CPE) 72 hours after infection. Stocks were considered to have passed this safety test when CPE was not detected in the final passage. Stocks were then removed from the BSL3 laboratory.

VRP neutralization and GFP reporter assay

Vero 81 cells (2.25×10^3 cells/well) were seeded into wells of 384-well plates and incubated at 37°C for 24 hours. Neat hybridoma supernatant or serial dilutions of purified mAbs were incubated with VRPs at an MOI of ~ 5 infectious units/cell in virus dilution buffer (VDB; RPMI medium containing 20 mM HEPES supplemented to contain 1% FBS) at 37°C for 1 hour and then adsorbed to cells. Cells were incubated at 37°C for 18 hours, stained with Hoechst stain to label nuclei, and imaged using an ImageXpress Micro XL imaging system (Molecular Devices) at the Vanderbilt High-Throughput Screening Facility. Total and CHIKV-infected cells (marked by GFP expression) were quantified using MetaXpress software (Molecular Devices) in two fields of view per well. For each antibody, EC_{50} values with 95% confidence intervals were determined using nonlinear regression to fit separate logistic growth curves using the R statistics program (*5*).

Virus stocks prepared as antigen for ELISA

The infectious clone plasmid for CHIKV vaccine strain 181/25 (*6*, *7*) was linearized with NotI-HF and transcribed *in vitro* using an mMessage mMachine SP6 transcription kit (Life Technologies). Viral RNA was introduced into BHK21 cells by electroporation. Culture supernatants were harvested 24 hours later, clarified by centrifugation at 855 × g for 20 min, aliquoted, and stored at -80°C.

Virus capture ELISA for hybridoma screening

Antibody binding to virus particles was performed by coating assay plates with purified mouse mAb CHK-187 (*8*), prepared at 1 μg/mL in 0.1 M Na_2CO_3 and 0.1 M $NaHCO_3$ pH 9.3 binding buffer, was used to coat ELISA plates (Nunc 242757) and incubated at 4°C overnight. After incubating plates for 1 hour with blocking buffer (1% powdered milk and 2% goat serum in PBS with Tween 20 [PBS-T]), plates were washed five times with PBS-T and incubated with 25 μL of culture supernatant from BHK21 cell monolayers infected with CHIKV vaccine strain 181/25. After incubation at room temperature for 1 hour, plates were washed ten times with PBS, and 10 μL of B cell culture supernatant was added into 25 μL/well of blocking buffer. Plates were incubated at room temperature for 1 hour prior to washing five times with PBS-T. A secondary antibody conjugated to alkaline phosphatase (goat anti-human Fc; Meridian Life Science, W99008A) was applied at a 1:5,000 dilution in 25 μL/well of blocking buffer, and plates were incubated at room temperature for 1 hour. Following five washes with PBS-T, phosphatase substrate solution (1 mg/mL phosphatase substrate in 1 M Tris aminomethane [Sigma, S0942]) was added at 25 μL/well, and plates were incubated at room temperature for 2 hours before determining the optical density at 405 nm using a Biotek plate reader.

CHIKV-specific control human mAbs

In some assays, two previously described human CHIKV-specific mAbs, 5F10 and 8B10 (*9*), were used as positive controls. These mAbs were expressed in 293F cells (Invitrogen) following transfection with an IgG1

expression plasmid (Lonza) containing a sequence-optimized cDNA of the 5F10 and 8B10 antibody variable gene regions based on sequences provided by Cheng-I Wang and Alessandra Nardin (Singapore Immunology Network, A*STAR, Singapore).

ELISA for mAb binding to E2 protein

Recombinant CHIKV E2 ectodomain protein (corresponding to the CHIKV-LR2006 strain) was generated in *E. coli* as described (*8*) and adsorbed to microtiter plates (100 μL of a 2 μg/mL E2 protein solution in 0.1 M Na_2CO_3, 0.1 M $NaHCO_3$, and 0.1 % NaN_3 [pH 9.3]) at 4°C overnight. Plates were rinsed three times with PBS containing 0.05% Tween-20, and incubated at 37 °C for 1 hour with blocking buffer (PBS, 0.05% Tween-20, and 2% [w/v] of BSA). Primary human mAb (diluted to 10 μg/mL in blocking buffer) was added to wells at room temperature for 1 hour. Plates were rinsed three times with PBS containing 0.05% Tween-20, and secondary antibody (biotin-conjugated goat anti-human IgG [H and L chains] with minimal cross-reactivity to mouse serum proteins [Jackson ImmunoResearch Laboratories] diluted 1/20,000 in blocking buffer) and streptavidin-conjugated horseradish peroxidase (diluted in PBS with 0.05% Tween-20 [Vector Laboratories]) were added sequentially, each at room temperature for 1 hour with plate rinsing in between steps. After four rinses with PBS, plates were incubated at room temperature with 100 μL of TMB (3,3′,5,5′-tetramethylbenzidine) chromogenic substrate solution (Dako) for 5 min, and the reaction was stopped by addition of 2 N H_2SO_4. Product intensity was determined using an ELISA plate reader at an optical density of 450 nm.

Affinity measurements by surface plasmon resonance

Interactions of purified human mAbs and CHIKV proteins were analyzed kinetically using a Biacore T100 instrument as described (*10*). *For the intact IgG with soluble CHIKV p62-E1,* anti-human IgG antibodies (GE

Life Sciences) were immobilized onto a Series S CM5 chip and used to capture anti-CHIKV or control (hu-WNV E16) antibodies. The CHIKV p62-E1 was injected over the surface at 65 μL/min for 180 sec and allowed to dissociate for 1000 sec before regeneration with 3 M $MgCl_2$ between cycles. Some antibodies did not bind to the monomeric E1 protein, therefore we tested them for binding to VLPs. *For the kinetic measurements with the CHIKV VLP*, anti-mouse IgG antibodies (GE Life Sciences) were immobilized to capture a set of mouse anti-CHIKV antibodies with sub-nanomolar affinities, which were in turn used to capture the CHIKV VLPs. Anti-CHIKV IgG or Fab was injected over the chip surface at 65 μL/min for 180 sec and allowed to dissociate for 1000 sec before regeneration with 10 mM glycine pH 1.7 between cycles. All data were processed using the Biacore Evaluation Software (Version 1.1.1) and a global 1:1 Langmuir fit of the curves. Results were obtained from at least three independent experiments.

Focus reduction neutralization test (FRNT) with infectious CHIKV

To determine mAb breadth and neutralization potency, we used four representative strains with at least one representative from each CHIKV genotype, including one prototype virus from each of the three genotypes and also a strain from the current Caribbean outbreak. Serial dilutions of purified human mAbs were incubated with 100 focus-forming units (FFU) of CHIKV at 37°C for 1 hour. MAb-virus complexes were added to Vero cells in 96-well plates. After 90 min incubation, cells were overlaid with 1% (w/v) methylcellulose in Modified Eagle Media (MEM) supplemented to contain 2% FBS. Cells were incubated for 18 hours and fixed with 1% paraformaldehyde in PBS. Cells were incubated sequentially with 500 ng/mL of murine CHK-11 (*8*) and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG in PBS supplemented to contain 0.1% saponin and 0.1% bovine serum albumin (BSA). CHIKV-infected foci were visualized using TrueBlue peroxidase substrate (KPL) and quantified using an ImmunoSpot 5.0.37 macroanalyzer (Cellular Technologies Ltd). EC_{50}

values were calculated using nonlinear regression analysis after comparison to wells inoculated with CHIKV in the absence of antibody.

Biolayer interferometry competition binding assay

CHIKV-LR2006 E2 ectodomain protein containing a polyhistidine-tag (20 μg/mL) was immobilized onto Anti-Penta-His biosensor tips (ForteBio #18-5077) for 2 min. After determining the baseline signal in kinetics buffer (KB, 1X PBS, 0.01% BSA and 0.002% Tween 20) for 1 min, biosensor tips were immersed into wells containing primary antibody at a concentration of 100 μg/mL for 5 min and then immersed into wells containing competing mAbs at a concentration of 100 μg/mL for 5 min. The percent binding of the competing mAb in the presence of the first mAb was determined by comparing the maximal signal of the competing mAb applied after the initial mAb complex to the maximal signal of competing mAb alone. Antibodies were judged to compete for binding to the same site if maximum binding of the competing mAb was reduced to < 30% binding affinity alone. Antibodies were considered non-competing if maximum binding of the competing mAb was > 70% of non-competed binding. A level of 30-70% of non-competed binding was considered intermediate competition.

Mutagenesis epitope mapping

A CHIKV envelope protein expression construct (strain S27, Uniprot Reference #Q8JUX5) with a C-terminal V5 tag was subjected to alanine-scanning mutagenesis to generate a comprehensive mutation library. Primers were designed to mutate each residue within the E2, 6K, and E1 regions of the envelope proteins (residues Y326 to H1248 in the structural polyprotein) to alanine; alanine codons were mutated to serine (*12*). In total, 910 CHIKV envelope protein mutants were generated (98.5% coverage), sequence confirmed, and arrayed into 384-well plates. HEK-293T cells were transfected with the CHIKV mutation library in 384-well plates and incubated for 22 hours. Cells were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) in PBS plus

calcium and magnesium (PBS+/+) and stained with purified mAbs at 0.25 to 1.0 µg/mL or purified Fab fragments at 2.5 µg /mL diluted in 10% normal goat serum (NGS; Sigma). Primary antibody concentrations were determined using an independent immunofluorescence titration curve against wild-type CHIKV envelope proteins to ensure that signals were within the linear range of detection. Antibodies were detected using 3.75 µg/mL AlexaFluor488-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) in 10% NGS. Cells were washed twice with PBS without magnesium and calcium (PBS -/-) and resuspended in Cellstripper (Cellgro) with 0.1% BSA (Sigma). Mean cellular fluorescence was detected using a high-throughput flow cytometer (HTFC, Intellicyt). Antibody reactivity against each mutant clone was calculated relative to wild-type protein reactivity by subtracting the signal from mock-transfected controls and normalizing to the signal from wild-type-transfected controls. Amino acids were identified as required for mAb binding if the corresponding alanine mutant did not react with the test mAb but did react with other CHIKV antibodies. This counter-screen strategy facilitates the exclusion of mutants that are misfolded or have an expression defect (*13-15*). Amino acids required for antibody binding were visualized on the CHIKV envelope protein crystal structure (monomer PDB ID #3N41 and trimer PDB ID #2XFB) using PyMol software.

Pre- and post-attachment neutralization assays

Vero 81 cells (ATCC CCL-81; ~ 7.5×10^3 cells/well) were seeded into wells of 96-well plates and incubated at 37°C for ~ 24 hours. For pre-attachment assays, dilutions of mAb were prepared at 4°C in virus dilution buffer (VDB) and pre-incubated with VRPs at 4°C for 1 hour. Antibody-virus complexes were added to pre-chilled Vero 81 cells at 4°C for 1 hour. Non-adsorbed virus was removed by three washes with VDB, and cells were incubated in complete medium at 37°C for 18 hours. The post-attachment assay was performed similarly, except that an equivalent MOI of VRPs was first adsorbed to Vero 81 cells at 4 °C for 1 hour, unbound VRPs were removed by three washes with virus dilution buffer, and cells were incubated with pre-chilled VDB

containing serial dilutions of mAb at 4°C for 1 hour. Unbound mAbs were removed by three washes with VDB, and cells were incubated in complete medium at 37°C for 18 hours. Cells were stained, imaged, and analyzed as described for VRP neutralization assays, with four fields of view per well, yielding a total of ~ 800 to 1,000 cells analyzed for GFP expression per sample.

Fusion inhibition assays

Virus fusion with the plasma membrane was assessed using an FFWO assay (*16*). Vero 81 cells (~ 3.75×10^3 cells/well) were seeded into wells of 96-well plates and incubated at 37°C for ~ 24 hours. Cells were washed once with binding medium (RPMI 1640 supplemented to contain 1% FBS, 25 mM HEPES [pH 7.4] and 20 mM NH_4Cl to prevent infection through endosomal fusion) and incubated in binding medium at 4°C for 15 min. Inoculum containing VRPs was diluted in binding medium and incubated with cells at 4°C for 1 hour. Unbound VRPs were removed by two washes with binding medium. Serial dilutions of mAbs in VDB were incubated with cells at 4°C for 1 hour, and unbound mAb was removed by two washes with VDB. FFWO was induced by the addition of pre-warmed fusion medium (RPMI 1640, 1% FBS, 25 mM HEPES, and 30 mM succinic acid at pH 5.5) at 37°C for 2 min. In parallel wells, control medium (RPMI 1640, 1% FBS, 25 mM HEPES at pH 7.4) was added at 37°C for two min. The medium was removed and cells were incubated in DMEM supplemented to contain 5% FBS, 20 mM NH_4Cl (to ensure that infection occurred only through pH-dependent plasma membrane fusion), and 25 mM HEPES [pH 7.4]). At 18 hours post infection, cells were stained, imaged, and analyzed as described, with four fields of view per well, yielding a total of ~ 800 - 1,000 cells analyzed for GFP expression per sample.

***In vivo* protection studies in mice**

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols were approved by the Institutional Animal Care and Use Committee at Washington University School of Medicine (Assurance Number: A3381-01). *Ifnar*$^{-/-}$ mice were bred in pathogen-free animal facilities at Washington University School of Medicine, and infection experiments were performed in A-BSL3 facilities with the approval of the Washington University Animal Studies Committee. Footpad injections were performed under anesthesia that was induced and maintained with ketamine hydrochloride and xylazine. For prophylaxis studies, human mAbs were administered by intraperitoneal injection to 6 week-old *Ifnar*$^{-/-}$ mice 1 day prior to subcutaneous inoculation in the footpad with 10 FFU of CHIKV-LR diluted in HBSS with 1% heat-inactivated FBS. For therapeutic studies, 10 FFU of CHIKV-LR was delivered 24, 48, or 60 hours prior to administration of a single dose of individual or combinations of human mAbs at specified doses.

Measurements of *in vivo* half life of mAbs in mice

We considered whether varying *in vivo* half-life in the recipient mice might account for the observed differences in efficacy of the human mAbs. We tested the half-life of mAbs 4B8, 5M16, 4N12, and 4J21 by injecting groups of 3-5 naïve mice with mAb, and then obtaining serum samples at 2 h, or 2, 4, or 8 d after injection using a human IgG capture ELISA and a capture ELISA using CHKV particles. There was no appreciable difference in the rate of clearance in the serum for any of the mAbs, and none of the mAb levels were decreased by 50% at 8 d indicating an extended half-life in these animals

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