

Expression, Purification, and Refolding of Chikungunya Virus Full-Length Envelope E2 Protein along with B-Cell and T-Cell Epitope Analyses Using Immuno-Informatics Approaches

Manisha Shukla, Pankaj Chandley, Suman Tapryal, Narendra Kumar, Sulakshana P. Mukherjee, and Soma Rohatgi*



Cite This: *ACS Omega* 2022, 7, 3491–3513



Read Online

ACCESS |



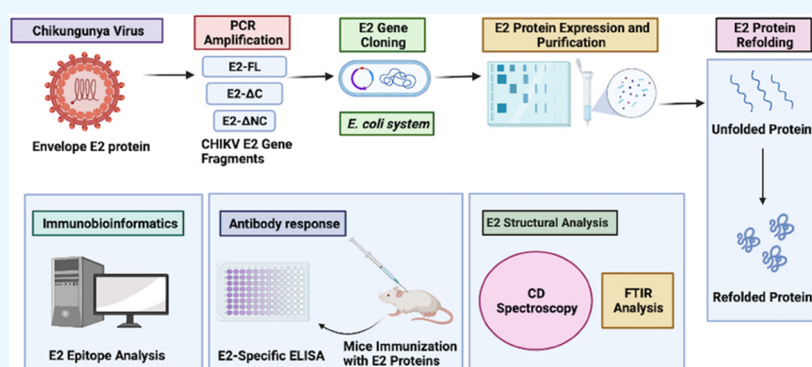
Metrics & More



Article Recommendations



Supporting Information



ABSTRACT: Chikungunya virus (CHIKV) is a mosquito-transmitted alphavirus, which causes severe illness in humans and is responsible for epidemic outbreaks in Africa, Asia, North and South America, and Europe. Despite its increased global prevalence, no licensed vaccines are available to date for treating or preventing CHIKV infection. The envelope E2 protein is one of the promising subunit vaccine candidates against CHIKV. In this study, we describe successful cloning, expression, and purification of CHIKV E2 full-length (E2-FL) and truncated (E2-ΔC and E2-ΔNC) proteins in the *Escherichia coli* expression system. The recombinant E2 proteins were purified from inclusion bodies using Ni-NTA chromatography. Further, we describe a detailed refolding procedure for obtaining the CHIKV E2-FL protein in native conformation, which was confirmed using circular dichroism and Fourier transform infrared spectroscopy. BALB/c mice immunized with the three different E2 proteins exhibited increased E2-specific antibody titers compared to sham-immunized controls, suggesting induction of strong humoral immune response. On analyzing the E2-specific antibody response generated in immunized mice, the CHIKV E2-FL protein was observed to be the most immunogenic among the three different CHIKV E2 antigens used in the study. Our B-cell and T-cell epitope mapping results indicate that the presence of specific immunogenic peptides located in the N-terminal and C-terminal regions of the CHIKV E2-FL protein may contribute to its increased immunogenicity, compared to truncated CHIKV E2 proteins. In summary, our study provides a detailed protocol for expressing, purifying, and refolding of the CHIKV E2-FL protein and provides an understanding of its immunogenic epitopes, which can be exploited for the development of novel multi-epitope-based anti-CHIKV vaccine strategies.

1. INTRODUCTION

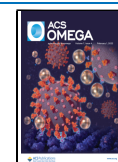
Chikungunya is a viral disease caused by chikungunya virus (CHIKV). CHIKV is a single-stranded RNA virus belonging to the *Togaviridae* family and genus *Alphavirus*.¹ It is transmitted to humans by infected *Aedes aegypti* and/or *Aedes albopictus* mosquitoes.^{2,3} Apart from mosquito transmission, some of the recent epidemics have arisen from maternal-fetal transmission as well.⁴ Chikungunya virus (CHIKV) causes severe illness and fever in humans, which is associated with other symptoms like rashes, myalgia, headache, and debilitating polyarthralgia.⁵ Although chikungunya fever is generally considered as self-limiting and nonfatal, casualties and complications have been seen in patients with comorbidities.⁶ While the acute stage of

chikungunya infection typically lasts for only 1–2 weeks, severe joint pain, critical morning stiffness, swelling, chronic fatigue, and consistent inflammatory arthritis may persist for weeks, months, or years in affected individuals.⁷ The laboratory diagnosis of CHIKV infection is based on viral RNA detection through RT-PCR and the presence of IgM and IgG antibodies

Received: October 26, 2021

Accepted: December 10, 2021

Published: January 14, 2022



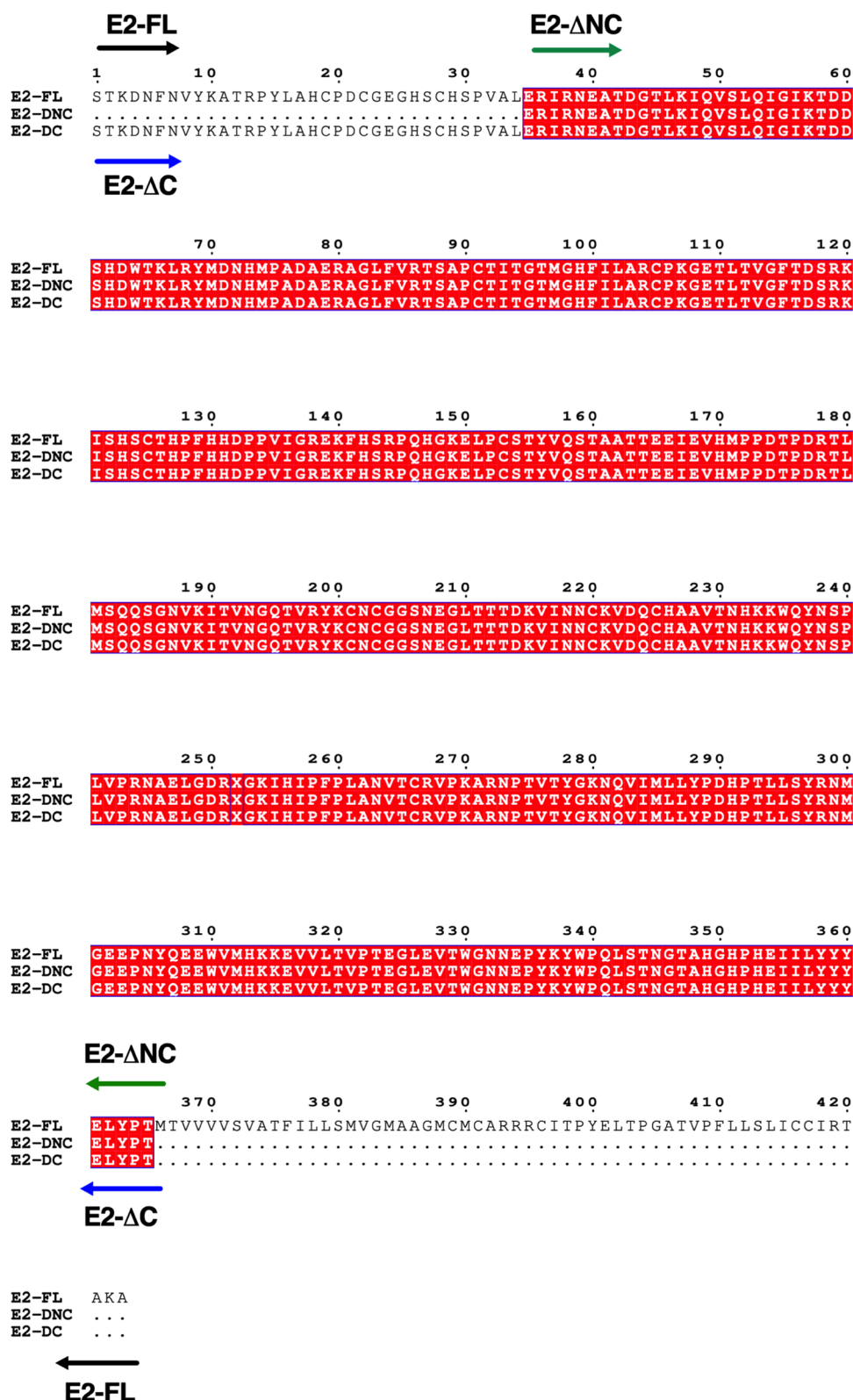


Figure 1. Amino acid sequence alignment of the CHIKV E2-FL and truncated E2 protein fragments used in the study. The 5' start and 3' stop regions for E2-FL (1–423 aa), E2-ΔC (1–365 aa), and E2-ΔNC (35–365 aa) proteins are indicated using black, blue, and green arrows, respectively.

through serological tests.⁸ Several studies have also reported the recurrence of the disease in patients infected with CHIKV after the initial infection or relapse of chikungunya infection.^{9,10} The case fatality rate has been estimated to be

1 in 1000, with most deaths occurring in newborn children, the elderly, and adults with underlying medical conditions.^{11–13} CHIKV was originally isolated in 1953 from the Makonde plateau in Tanzania, followed by numerous outbreaks in Africa

and Asia.^{14–16} Globally, the viral transmission is responsible for epidemic outbreaks in Africa, Europe, Southeast Asia, India, North America, and South America.¹⁷ In spite of its geographical expansion and outbreak, no specific treatment and effective licensed vaccines are currently available to prevent CHIKV infection. Hence, there is a need to understand and develop anti-CHIKV immunity through either vaccination or passive immunization strategies.

CHIKV is a spherical alphavirus, consisting of an enveloped particle comprising a nucleocapsid core containing positive-sense single-stranded RNA as a genetic material. The CHIKV genome consists two long open reading frames (ORFs). The genome of CHIKV (approximately 12 kb) encodes for five structural (envelope proteins E1, E2, E3, capsid, and 6K) and four nonstructural (nsP1–4) proteins. The E1 and E2 glycoproteins are mainly responsible for membrane fusion and virus entry into host cells, where E2 interacts with the cellular receptor and functions in attachment to cells and E1 participates in virus fusion to the cell membrane.¹⁸ The E2 protein contains three distinct domains: A (16–134 aa), B (173–231 aa), and C (269–341 aa), which are involved in receptor binding and are considered immunogenic.^{19,20} Literature reports show increasing evidence for the importance of antibody-mediated protection against CHIKV, and E2 glycoprotein has been implicated as the main target for the anti-CHIKV antibody response during the entire course of disease (from the convalescent phase to the recovery phase).^{21,22} Neutralizing antibodies raised against the E2 protein are believed to be crucial for protection in animal models.^{23,24} Since humoral immunity (conferred by B cells and antibodies) is an essential component of protective host response against CHIKV,²⁵ passive immunotherapy using E2-specific monoclonal antibodies as well as polyclonal human immunoglobulin (Ig) has been explored with considerable success in CHIKV infection.²⁶ Among some of the diverse vaccine strategies being explored for CHIKV, including live-attenuated or whole-inactivated virus, recombinant virus-vectored vaccines, inactivated viruslike particles (VLPs), nucleic acid vaccines, and subunit vaccine formulations focusing on the CHIKV envelope E2 protein have shown various advantages like safety, efficacy, scalable production, and cost-effectiveness during development.^{27,28}

In the present study, we describe the cloning, expression, and purification of the CHIKV E2-FL protein along with its two truncated versions using an *Escherichia coli*-based prokaryotic expression system. The CHIKV E2-FL and truncated proteins were solubilized from inclusion bodies and purified using Ni-NTA chromatography. The E2 proteins were successfully refolded using a multistep dialysis procedure, and a secondary structure of refolded CHIKV E2 proteins was confirmed by circular dichroism (CD) and Fourier transform infrared (FTIR) spectroscopies. On testing the immunogenic nature of E2 proteins, the E2-FL protein was seen to exhibit significantly higher antibody titers in immunized mice when compared with sham-immunized controls. Our results from B-cell and T-cell epitope analyses using CHIKV E2-FL and truncated proteins demonstrate that specific epitopes located exclusively in the N-terminal and C-terminal regions of the CHIKV E2-FL protein may confer increased immunogenicity and provides insights into the development of a multi-epitope and/or multivalent anti-CHIKV vaccine. In summary, the simplicity and cost-effectiveness of the recombinant CHIKV E2-FL protein purification methodology described herein are

not only important for resource-limiting conditions but also can be exploited for developing fast and effective CHIKV E2-protein-based diagnostic or serological assays and can be utilized further in designing novel vaccine strategies.

2. RESULTS

2.1. Sequence Alignment of Full-Length and Truncated E2 Proteins. Sequence analysis of CHIKV E2-FL and E2 truncated proteins was performed using Clustal software. The amino acid sequence alignment of the CHIKV E2-FL and truncated protein fragments used in this study is shown in Figure 1. The regions for CHIKV E2-FL (1–423 aa), E2- Δ C (1–365 aa), and E2- Δ NC (35–365 aa) are indicated using black, blue, and green arrows, respectively. The CHIKV E2 protein (1–423 aa) contains a total of 17 cysteine residues, which may cause protein aggregation.²⁹ Therefore, besides the CHIKV E2-FL protein, we also synthesized two truncated E2 protein fragments, namely, (a) the E2- Δ C (1–365 aa) fragment missing five cysteine residues from the C-terminal and (b) the E2- Δ NC (35–365 aa) fragment, missing five cysteine residues from the C-terminal and an additional three cysteine residues from the N-terminal end. While the E2- Δ C (1–365 aa) fragment had a truncation of 58 aa from the C-terminal portion, the E2- Δ NC (35–365 aa) fragment had a truncation of 58 aa from the C-terminal portion as well as 34 aa from the N-terminal end (330 aa). Figure 2 shows a

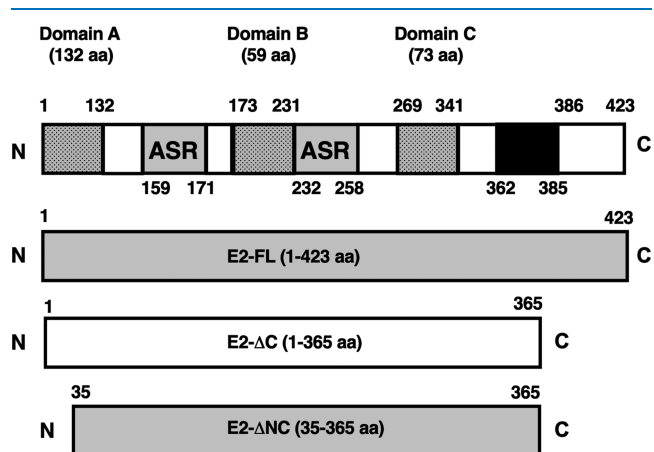


Figure 2. Schematic representation of full-length and truncated CHIKV E2 proteins used in the study. The three different E2 domains (dotted gray boxes) are indicated on top. The C-terminal transmembrane region (362–385 aa) is depicted in black. ASR, acid-sensitive regions (gray boxes). The C-terminal deleted (E2- Δ C) protein fragment (1–365 aa) and both C-terminal and N-terminal deleted (E2- Δ NC) protein fragment (35–365 aa) are outlined below the full-length E2 protein (1–423 aa).

schematic representation of the full-length and truncated CHIKV E2 fragments used in the study. CHIKV E2-FL contains three structural domains, Domain A (132 aa, spanning 1–132 aa), Domain B (59 aa, spanning 173–231 aa), and Domain C (73 aa, spanning 269–341 aa), two acid-sensitive regions (ASRs; from 159–171 aa and from 232–258 aa), and a 23 aa-long C-terminal transmembrane region (spanning 362–385 aa).^{20,21}

2.2. PCR Amplification and Cloning of Full-Length and Truncated E2 Proteins. Total RNA was extracted from the chikungunya viral lysate (Ind-06-Guj), and virus cDNA was synthesized using the oligo-dT primer as mentioned in

Table 1. Primer Sequences Used for PCR Amplification and Generation of Recombinant Full-Length and Truncated CHIKV E2 Fragments

E2-regions	primers used	primer sequence ^c	base pairs	position	amino acids ^d
CHIKV E2-FL	E2-FL FP	CCCCGGATCCAGCACCAAGGACAACCTCAATG	1269 bp	1–423 aa	423 aa
	E2-FL RP	CCCCCAAGCTTCGCTTTAGCTGTTCTGATGCAG			
CHIKV E2-ΔC ^a	E2-FL FP	CCCCGGATCCAGCACCAAGGACAACCTCAATG	1095 bp	1–365 aa	365 aa
	E2-TC RP	CCCCCAAGCTTAGTAGGGTACAGCTCATAATAATAC			
CHIKV E2-ΔNC ^b	E2-TNC FP	CCCCGGATCCGAACGCATCAGAAATGAAGCGAC	993 bp	35–365 aa	331 aa
	E2-TC RP	CCCCCAAGCTTAGTAGGGTACAGCTCATAATAATAC			

^aThe CHIKV E2-ΔC fragment was obtained using listed forward and reverse primers and the CHIKV E2-FL cloned plasmid as the template. ^bThe CHIKV E2-ΔNC fragment was obtained using listed forward and reverse primers and the CHIKV E2-FL cloned plasmid as the template. ^cThe underlined nucleotides in forward and reverse primer sequences represent *Bam*HI and *Hind*III restriction enzyme sites, respectively. ^dBesides the corresponding amino acids for CHIKV E2 fragments (shown in bold), all constructs included additional 31 aa from the vector (28 aa from the N-terminal and 3 aa from the C-terminal).

Experimental section. The gene-specific primers designed for amplifying E2-FL, E2-ΔC, and E2-ΔNC fragments are outlined in Table 1. The full-length CHIKV E2 gene (referred to as E2-FL) corresponding to 1269 bp was PCR-amplified using CHIKV cDNA and gene-specific primers as mentioned in the Experimental section (Figure 3A). PCR reactions for

ΔNC positive clones showed fallout of the correct size insert genes. Sanger sequencing results showed no mutation or reading-frame shift in any of the CHIKV E2 fragments used in this study.

2.3. Expression of Full-Length and Truncated E2 Proteins. The *E. coli* SG13009 (Qiagen)-competent cells were transformed with recombinant CHIKV E2-FL, E2-ΔC, and E2-ΔNC plasmids in the pQE-30 Xa vector backbone carrying an N-terminal 6-His tag. The CHIKV E2-FL and truncated proteins were expressed in *E. coli* cells under IPTG induction. Next, overexpression of recombinant CHIKV E2-FL, E2-ΔC, and E2-ΔNC proteins under IPTG induction was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The molecular weights of each of the three proteins, viz. E2-FL, E2-ΔC, and E2-ΔNC, were calculated using online tools and were found to be approximately 50, 44, and 40 kDa, respectively. SDS-PAGE gels stained with Coomassie brilliant blue staining show the presence of distinct bands migrating alongside the molecular weight marker bands, corresponding to their calculated molecular weights (Figure 4). Specific bands were obtained for histidine-tagged CHIKV E2 fusion proteins corresponding to E2-FL (50 kDa, Figure 4A), E2-ΔC (44 kDa, Figure 4B), and E2-ΔNC (40 kDa, Figure 4C). To investigate whether the full-length and truncated E2 proteins were expressed in the soluble or insoluble form inside *E. coli*, bacterial cultures obtained after IPTG induction were centrifuged. Bacterial pellets were subsequently analyzed on SDS-PAGE gels. The expressed CHIKV E2-FL and truncated proteins were found in the insoluble fraction (inclusion bodies), which was processed for further purification steps.

2.4. Purification of Full-Length and Truncated E2 Proteins. The T5 promoter/lac operator transcription-translation system-based pQE-30 Xa vector with an N-terminal 6-His tag offers an affinity-based purification strategy of recombinant fusion proteins expressed in *E. coli*. Nickel nitrilotriacetic acid (Ni-NTA) chromatography was chosen to purify the His-tagged CHIKV E2-FL and truncated proteins. Since the recombinant proteins were seen to accumulate in inclusion bodies, purification was performed under denaturing conditions in the presence of urea. The *E. coli* SG13009 cells harboring CHIKV E2-FL, E2-ΔC, and E2-ΔNC constructs were harvested from a 500 mL culture volume, and their cell weights were measured. The pellets were stored in Lysis buffer (300 mM NaCl, 25 mM Tris pH 7.3, 8 M urea, 5 mM β-ME, 0.1% Triton X-100, 10% glycerol) along with 1 mM PMSF and proteinase inhibitor cocktail at –80 °C overnight. The cells were then lysed using a Lysis buffer supplemented with

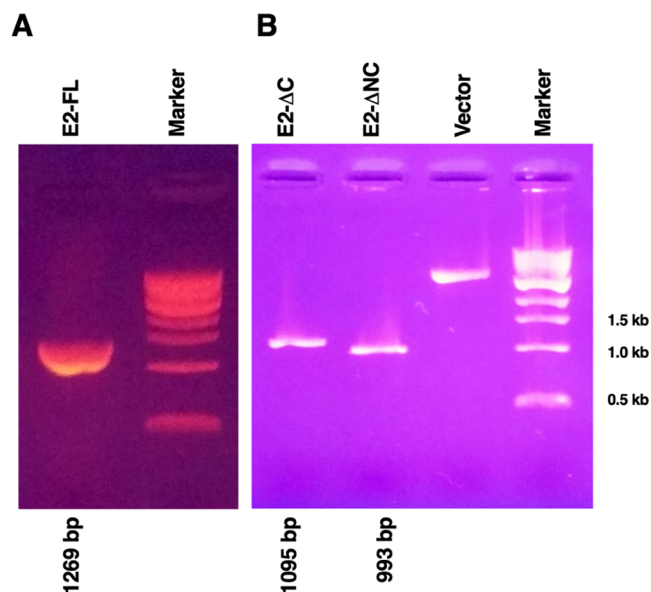


Figure 3. PCR amplification of CHIKV E2-FL and truncated fragments used in the study. (A) PCR amplification of the CHIKV E2 full-length gene (1269 bp). (B) PCR amplification of truncated E2 fragments, E2-ΔC (1095 bp), and E2-ΔNC (993 bp). Vector: linearized pQE-30 Xa vector. Marker: 1 Kb DNA ladder; last three marker bands are indicated on the right.

amplifying the two different truncated E2 fragments were carried out using the full-length E2 gene as a template. Single specific bands corresponding to 1095 bp and 993 bp were obtained for truncated CHIKV E2-ΔC and E2-ΔNC gene fragments, respectively (Figure 3B). The PCR amplicons were subjected to double digestion with restriction enzymes (*Bam*H1 and *Hind*III) and cloned into the digested pQE-30 Xa vector having corresponding overhangs for enabling directional cloning. The ligated products were transformed in the *E. coli* XL1-Blue strain. The colonies obtained after transformation were subjected to plasmid isolation, and positive clones were identified by restriction analysis and confirmed by sequencing. The CHIKV E2-FL, E2-ΔC, and E2-

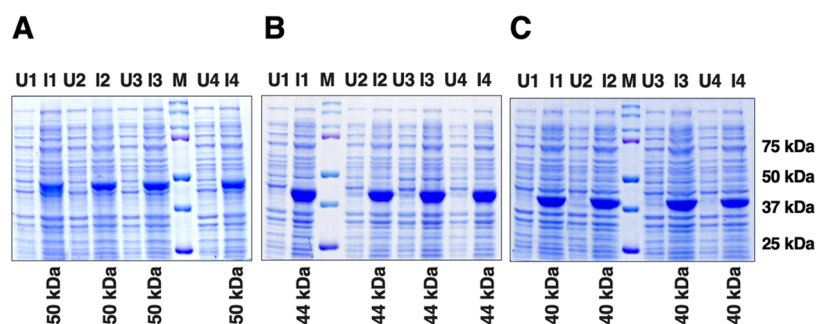


Figure 4. Protein induction of CHIKV E2 full-length and truncated fragments used in the study. (A) CHIKV E2 full-length protein (423 aa; ~50 kDa). (B) CHIKV E2 C-terminal truncated fragment E2- Δ C (365 aa; ~44 kDa). (C) CHIKV E2 N-terminal and C-terminal truncated fragment E2- Δ NC (331 aa; ~40 kDa). Lanes U: uninduced samples; Lanes I: induced samples; M: protein marker; marker bands are indicated on the right.

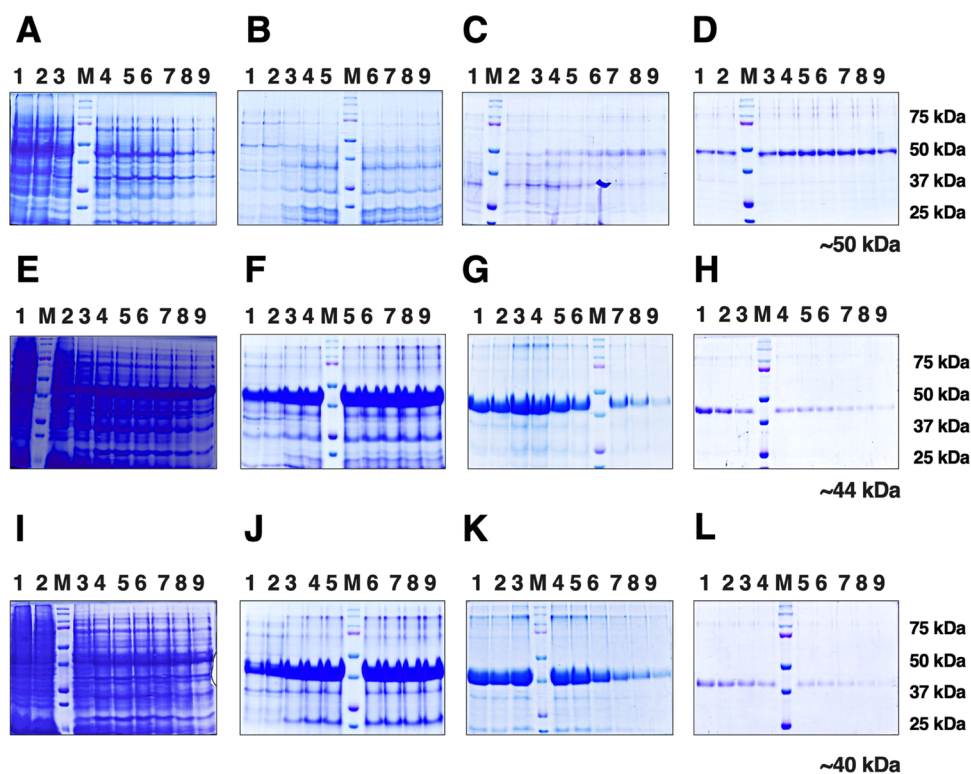


Figure 5. SDS-PAGE analysis of purified full-length and truncated His-tagged CHIKV E2 proteins used in the study. Fractions of purified proteins obtained under denaturing conditions (8.0 M urea, pH 7.3) using different imidazole concentrations in washed and elution buffers: Gels A, E, and I: 20 mM imidazole; gels B, F, and J: 50 mM imidazole; gels C, G, and K: 100 mM imidazole; and gels D, H, and L: 250 mM imidazole. (A–D) CHIKV E2 full-length protein (423 aa; ~50 kDa). (E–H) CHIKV E2 C-terminal truncated fragment E2- Δ C (365 aa; ~44 kDa). (I–L) CHIKV E2 N-terminal and C-terminal truncated fragment E2- Δ NC (331 aa; ~40 kDa). Lanes 1–9: washed or eluted samples obtained at a given buffer concentration; M, protein marker; marker bands are indicated on the right.

lysozyme and mechanical sonication followed by syringe passing. The inclusion bodies were extracted by centrifugation, and the supernatants obtained were incubated with Ni-NTA slurry (Qiagen) and loaded into columns. After obtaining flowthrough, the columns were washed with a lysis buffer supplemented with 20 mM, 50 mM, and 100 mM imidazole. Finally, the His-tagged fusion proteins were eluted using a lysis buffer supplemented with 250 mM imidazole. During column washing steps in Ni-NTA chromatography, wash buffers were supplemented with 0.1% Triton X-114 for removing endotoxin contamination in purified CHIKV E2 proteins.

The purities of CHIKV E2-FL and E2 truncated protein fractions were estimated by SDS-PAGE electrophoresis, wherein the purified E2-FL and truncated proteins migrated

through denaturing SDS-PAGE gels according to their expected molecular weight and were visualized using Coomassie brilliant blue staining. Figure 5 shows the SDS-PAGE analysis of washed and eluted fractions obtained under denaturing conditions (8.0 M urea, pH 7.3) using different imidazole concentrations for histidine-tagged CHIKV E2-FL, E2- Δ C, and E2- Δ NC proteins used in this study. For purifying the CHIKV E2-FL protein, the column was initially washed with 20 mM imidazole wash buffer and the fractions obtained are shown in lanes 1–9 (Figure 5A). Bacterial impurities in CHIKV E2-FL protein preparation were further removed by washing the column with 50 mM imidazole wash buffer, as indicated in lanes 1–9 (Figure 5B). To further purify the CHIKV E2-FL protein, columns were washed with 100 mM

imidazole wash buffer, as shown in lanes 1–9 (Figure 5C). Subsequently, the imidazole concentration was increased to 250 mM in the elution buffer for eluting the CHIKV E2-FL protein (Figure 5D). The purified CHIKV E2-FL protein was seen to correspond to ~50 kDa size as per the protein marker. For purifying the CHIKV E2-ΔC protein, the column was washed with 20 mM imidazole wash buffer, and the fractions obtained are shown in lanes 1–9 (Figure 5E). On using wash buffer supplemented with 50 mM imidazole, fractions containing impurities were observed in lanes 1–9 (Figure 5F). The bacterial impurities were further removed by washing the column with 100 mM wash buffer, and the fractions obtained are shown in lanes 1–9 (Figure 5G). Subsequently, the column was then passed with a 250 mM imidazole elution buffer for eluting the CHIKV E2-ΔC protein fractions shown in lanes 1–9 (Figure 5H). The purified CHIKV E2-ΔC protein corresponded to ~44 kDa size as per the protein marker. For purifying the CHIKV E2-ΔNC protein, the Ni-NTA column was washed with wash buffer containing 20 mM imidazole, and the fractions obtained are depicted in lanes 1–9 (Figure 5I). Next, the column was washed with wash buffer containing 50 mM imidazole, and the fractions obtained are shown in lanes 1–9 (Figure 5J). Bacterial impurities were removed by passing the column with wash buffer containing 100 mM imidazole elution buffer, as shown in lanes 1–9 (Figure 5K). Finally, the CHIKV E2-ΔNC protein was eluted by passing the column with elution buffer containing 250 mM imidazole, and fractions obtained are shown in lanes 1–9 (Figure 5L). The purified CHIKV E2-ΔNC protein corresponded to ~40 kDa size as per the protein marker.

2.5. Western Blotting of Full-Length and Truncated E2 Proteins. After purification, a single specific band corresponding to 50 kDa, 44 kDa, and 40 kDa was observed for CHIKV E2-FL, E2-ΔC, and E2-ΔNC proteins, devoid of any bacterial impurities. To confirm that the 50 kDa, 44 kDa, and 40 kDa bands observed on SDS-PAGE gels were of CHIKV E2-FL, E2-ΔC, and E2-ΔNC proteins, a western blot hybridization was performed using the anti-His antibody (Figure 6). The band sizes at 50 kDa, 44 kDa, and 40 kDa, which correspond to His-tagged CHIKV E2-FL, E2-ΔC, and E2-ΔNC fusion proteins were detected, suggestive of the successful cloning, expression, and purification of CHIKV E2-FL and truncated proteins. CHIKV E2 protein estimation was performed using the BCA assay as per the commercially available kit. The physicochemical properties of CHIKV E2-FL, E2-ΔC, and E2-ΔNC proteins were predicted by the ProtParam analysis tool on the ExPasy website (Table 2).

2.6. Refolding of Full-Length and Truncated E2 Proteins. After protein purification with Ni-NTA chromatography, refolding of the denatured protein was attempted using the direct refolding (single-step) or stepwise refolding (multistep) dialysis procedure. In the direct refolding method, purified and truncated E2 protein fractions were dialyzed against the refolding buffer (containing 20 mM Tris pH 7.3, 400 mM NaCl, 5 mM EDTA, and 20% glycerol) at pH 7.3 for 6 h at 21 °C. After 6 h, a visible protein precipitate was observed. For the improvement of the refolding efficiency of CHIKV E2-FL and E2 truncated proteins, we modified and further optimized the refolding procedure. Instead of decreasing the urea concentration in a single step, we optimized the refolding of CHIKV E2-FL and E2 truncated proteins by decreasing the urea concentration in a total of 10 steps. Figure 7 shows a schematic representation of the

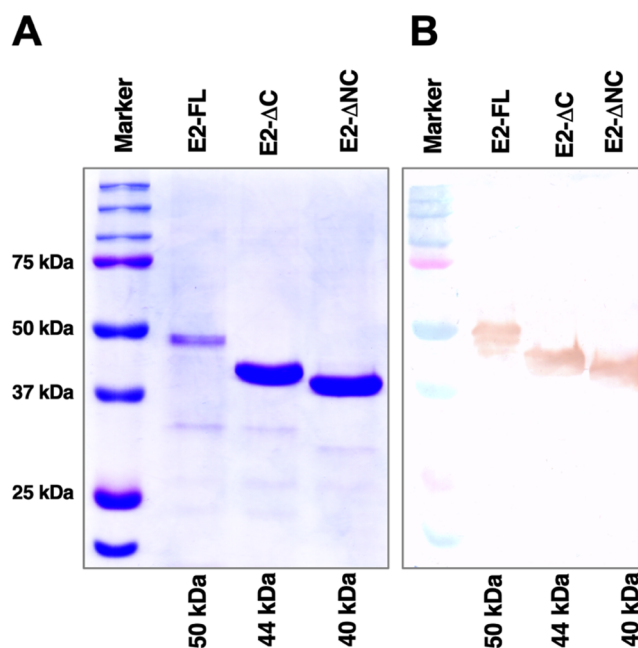


Figure 6. Western blotting of full-length and truncated CHIKV E2 proteins used in the study. (A) SDS-PAGE using Coomassie brilliant blue staining. (B) Western blot using the DAB substrate. Histidine-tagged full-length (E2-FL, ~50 kDa), C-terminal truncated E2 (E2-ΔC, ~44 kDa), and both N-terminal and C-terminal truncated E2 (E2-ΔNC, ~40 kDa) proteins are shown. Protein marker band sizes are indicated on the left.

Table 2. Analysis of Physicochemical Properties of Full-Length and Truncated CHIKV E2 Protein Fragments Using the ProtParam Server

criteria	E2-FL	E2-ΔC	E2-ΔNC
number of amino acids	454	396	362
molecular weight (Da)	50 523.73	44 359.06	40 657.94
theoretical pI	8.48	7.97	8.05
number of negative amino acids (Asp +Glu)	40	39	36
number of positive amino acids (Arg +Lys)	46	41	38
instability index	36.66 (stable protein)	35.45 (stable protein)	34.51 (stable protein)
grand average of hydrophobicity (GRAVY)	-0.462 (hydrophilic protein)	-0.710 (hydrophilic protein)	-0.722 (hydrophilic protein)

detailed refolding procedure of CHIKV E2-FL and truncated proteins used in this study.

In the stepwise refolding method, the purified CHIKV E2-FL and E2 truncated protein fractions were dialyzed against the refolding buffer (containing 20 mM Tris pH 7.3, 400 mM NaCl, 5 mM EDTA) at pH 7.3 for 3 h. The protein fractions were dialyzed with 6 M urea at 21 °C for 3 h. Subsequently, the E2 protein fractions were subjected to refolding steps in 4 and 2 M urea at 21 °C for a time duration of 3 h each. The protein samples in 2 M urea were diluted to a final concentration of 1 M urea by addition of an equal volume of urea-free dialysis buffer, followed by dialysis against a buffer containing 1 M urea for 3 h. After 3 h, the protein fractions were dialyzed with gradually decreasing concentrations of urea (0.5, 0.25, and 0.125 M) at 4 °C and a time duration of 3 h each. The urea concentration was further decreased gradually

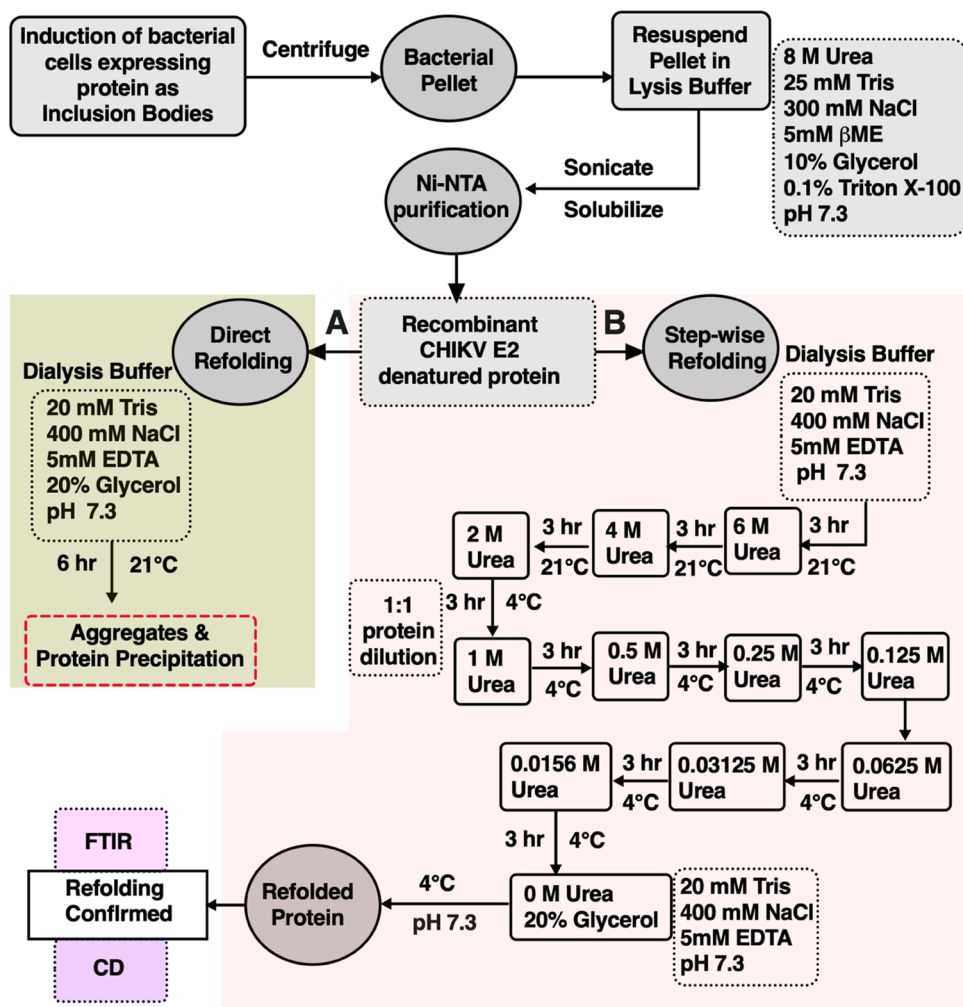


Figure 7. Schematic representation of the refolding procedure of CHIKV E2 full-length and truncated proteins after solubilizing inclusion bodies. (A) Direct refolding method involving single-step dialysis for the refolding of the CHIKV E2 denatured protein, which led to protein precipitation. (B) Stepwise refolding method involving the multistep dialysis procedure optimized for the refolding of full-length and truncated CHIKV E2 denatured protein samples.

to 0.0625, 0.03125, and 0.0156 M concentrations, and protein fractions were dialyzed at 4 °C and a time duration of 3 h each. Finally, the CHIKV E2 protein fractions were subjected to dialysis in a urea-free (0 M urea) dialysis buffer (20 mM Tris pH 7.3, 400 mM NaCl, 5 mM EDTA, and 20% glycerol) at pH 7.3 and 4 °C for 3 h with continuous stirring to obtain the refolded CHIKV E2-FL and truncated protein fragments.

We observed that the direct refolding method for the refolding of the CHIKV E2 unfolded protein in urea led to formation of protein precipitates. On the other hand, by optimizing a stepwise refolding method for the refolding of CHIKV E2-FL and E2 truncated protein samples, no visible protein precipitates were observed. Both unfolded and refolded protein fractions were used for subsequent structural analysis.

2.7. Structural Analysis of Full-Length and Truncated E2 Proteins. We used CD spectroscopy to analyze the secondary structure of denatured and refolded CHIKV E2-FL and truncated proteins used in the study. The CD spectral data were analyzed and deconvoluted using the CD spectra algorithm available online, as mentioned in the Methods section (Figure 8). Molar ellipticity is depicted on the y-axis with wavelength on the x-axis. The CD spectra were compared for denatured and refolded forms of CHIKV E2-FL (Figure

8A), E2- Δ C (Figure 8B), and E2- Δ NC (Figure 8C) proteins. On comparing CD spectra for each of the refolded and denatured CHIKV E2-FL and truncated protein samples, a change in CD spectra was observed in the folded sample when compared with the denatured samples. As shown in Figure 8A–C, the dip seen around 214 nm in the CD spectra for the refolded protein samples suggests the presence of secondary structure elements, especially β -strands,³⁰ whereas the denatured samples depicted a predominantly random coil structure.

To further affirm the correctly folded state of the *in vitro* refolded E2 proteins, the respective samples of CHIKV E2-FL (1–423 aa), E2- Δ C (1–365 aa), and E2- Δ NC (35–365 aa) were subjected to FTIR spectroscopy. The IR spectral data of proteins is usually interpreted in terms of vibrations of secondary structure repeat units, which give rise to nine absorption bands, namely, amides A, B, and I–VII. Among these, the amide-I band (1700–1600 cm^{-1}), which is generated by the C=O stretch vibrations of the peptide linkages, is the most useful in predicting the secondary structure of proteins. The amide bands in the regions of 1642–1624 and 1696–1691 cm^{-1} correspond to β -sheets, whereas the amide bands in the regions of 1656–1651 and 1685–1667

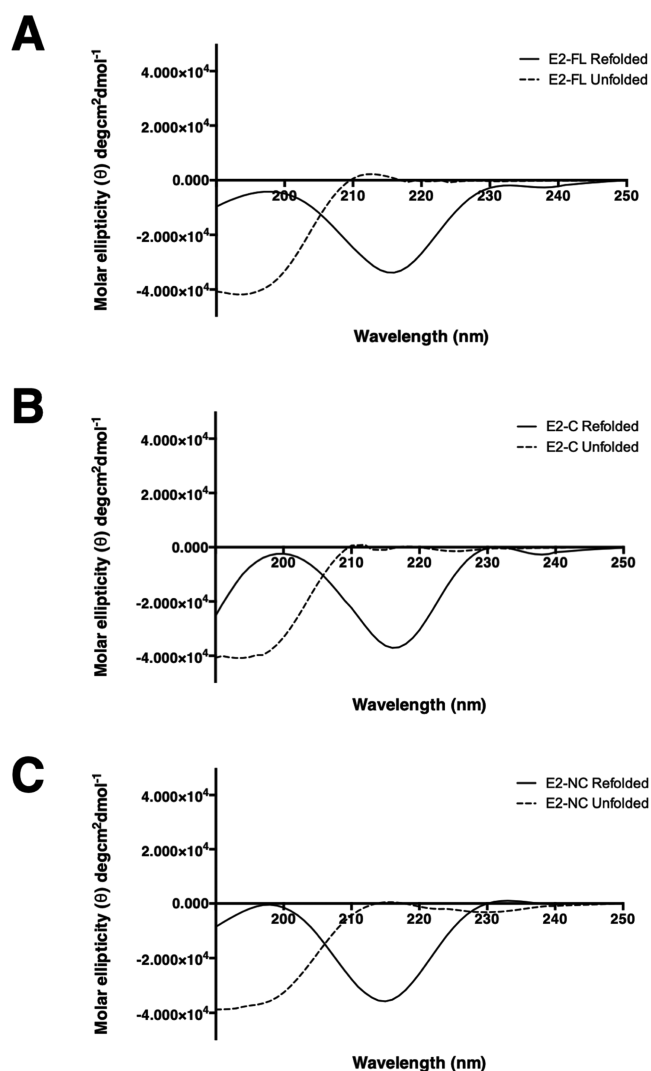


Figure 8. Far UV-CD spectra analysis of denatured and refolded CHIKV E2 full-length and truncated proteins. CD spectra obtained for (A) E2-FL, (B) E2- Δ C (B), and (C) E2- Δ NC proteins are shown. Molar ellipticity is depicted on the y -axis with wavelength on the x -axis. The denatured and refolded samples are shown in dotted lines and black lines, respectively.

cm^{-1} correspond to α -helix and β -turn secondary structures, respectively.³¹ In the case of each refolded E2 protein sample, a sharp and distinct amide band was observed at 1633/1635 cm^{-1} corresponding to β -sheet structures (Figure 9).

The FTIR results obtained for unfolded E2-FL, E2- Δ C, and E2- Δ NC proteins are shown in Figure S1. The peaks obtained at 1633/1635 cm^{-1} for unfolded E2 protein samples were not as sharp compared to the peaks obtained for refolded protein samples. The spectral data are depicted for refolded CHIKV E2-FL (Figure 9A), E2- Δ C (Figure 9B), and E2- Δ NC (Figure 9C) proteins. This spectral data also correlate with the crystal structures of the E2 protein reported earlier, wherein it has been shown that all three domains of the E2 protein are composed of β -sandwich motifs.¹⁹ Therefore, the CD and FTIR data collectively suggest that the refolded CHIKV E2-FL, E2- Δ C, and E2- Δ NC proteins have attained their native conformations.

2.8. Antibody Immune Responses against Full-Length and Truncated E2 Proteins. To evaluate the

immunogenic efficiencies of CHIKV E2-FL, E2- Δ C, and E2- Δ NC proteins, different groups of wild-type BALB/c mice were immunized with CHIKV E2-FL and E2 truncated proteins along with alum as the adjuvant. Before mice immunization, endotoxin levels in purified CHIKV E2 proteins were quantified using a commercial kit and the endotoxin levels in all immunogens were found to be between 0.5 and 0.1 EU/mL. Serum obtained from CHIKV E2-immunized and sham-immunized mice was tested by ELISA to detect the presence of antibodies specific to the CHIKV E2-FL protein. The CHIKV E2-specific total Ig titers in different groups of mice immunized on day 0, day 14, and day 42 with CHIKV E2-FL, E2- Δ C, and E2- Δ NC antigens and sham controls are shown (Figure 10). Total Ig antibody levels in CHIKV E2-immunized mice were found to be increased compared to those in sham-immunized controls, suggesting the induction of strong humoral immune response. Compared with sham-immunized controls, mice immunized with the CHIKV E2-FL protein exhibited significantly increased Ig titers as opposed to mice immunized with E2- Δ C and E2- Δ NC proteins on days 7, 14, 21, 28, 35, 42, 49, and 56 post immunization. At each timepoint, no significant difference ($p > 0.05$) was noted when antibody titers for the three different CHIKV E2 proteins were compared. CHIKV E2-specific Ig titers increased significantly after each booster dose of the E2 antigen, with peak antibody titers observed on day 49, one week after the second booster dose. Of note, we found that E2-specific antibody titers obtained after CHIKV E2-FL immunization were consistently higher than antibody titers obtained after immunization with E2- Δ C and E2- Δ NC proteins, at all times examined. Sham-immunized mice had very low or negligible antibody titers, which indicate specific antibody response generated only against CHIKV E2 proteins in immunized mice. Our results demonstrate that although immunization with CHIKV E2-FL, E2- Δ C, and E2- Δ NC proteins could efficiently produce high antibody titers in mice, the CHIKV E2-FL protein was seen to be the most immunogenic among the three different E2 antigens used in the study, making it a potent immunogenic antigen against CHIKV infection.

2.9. Epitope Analysis of Full-Length and Truncated E2 Proteins. **2.9.1. B-Cell Epitope Analysis.** Tertiary (three-dimensional, 3D) structure modeling was performed using SWISS-MODEL (<https://swissmodel.expasy.org>) and I-TASSER (I-TASSER server for protein structure and function prediction (zhanggroup.org)) for all three CHIKV E2 protein fragments based on available structure templates. The predicted structures of the three CHIKV E2-FL, E2- Δ C, and E2- Δ NC proteins were visualized in PyMOL graphics (Figure 11).

To further identify the unique epitopes and investigate the immunogenicity difference in all three CHIKV E2 protein fragments used in this study, we performed B-cell epitope analysis using two different approaches. In the first approach, we analyzed the presence or absence of B-cell epitopes identified in previously published studies, which included experimental validation of these epitopes in the CHIKV E2-FL, E2- Δ C, and E2- Δ NC proteins. A number of previously published studies have experimentally mapped and identified CHIKV E2 protein B-cell epitopes in human, mouse, and nonhuman primates using synthetic overlapping peptides.^{24,28,32,33} On comparing the sequences of CHIKV E2-FL, E2- Δ C, and E2- Δ NC proteins for the presence or absence of 10 B-cell epitopes identified by Kam et al.,^{32,34} we found the

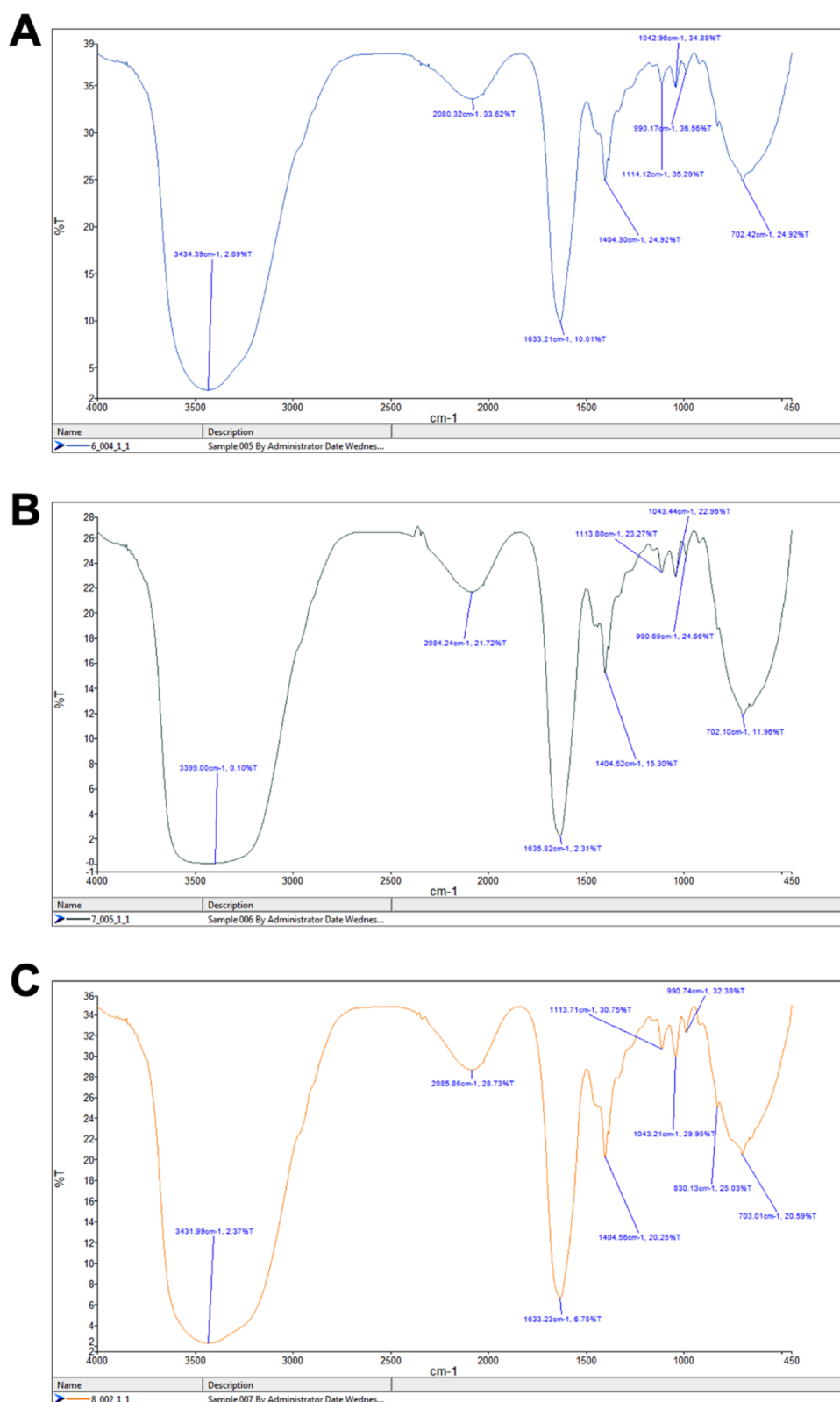


Figure 9. FTIR spectra of refolded CHIKV E2 full-length and truncated protein samples used in the study. (A) E2 full-length (1–423 aa), (B) E2- Δ C (1–365 aa), and (C) E2- Δ NC (35–365 aa) protein samples. In the FTIR spectra, the sharp and prominent peaks obtained at around 1633/1635 cm^{-1} indicate the presence of β -sheet-rich structures in the refolded protein samples.

presence of all 10 epitopes in the CHIKV E2-FL protein (Table 3). However, E2- Δ C and E2- Δ NC protein fragments showed the presence of only 9 epitopes and 8 epitopes, respectively. Specifically, the N-terminal epitope (1–19 aa)

was found absent in the E2- Δ NC protein fragment, while the C-terminal epitopes (378–411) were found absent in both the E2- Δ C and E2- Δ NC protein fragments (shown in blue and red, Figure 11). On comparing the sequences of CHIKV E2-

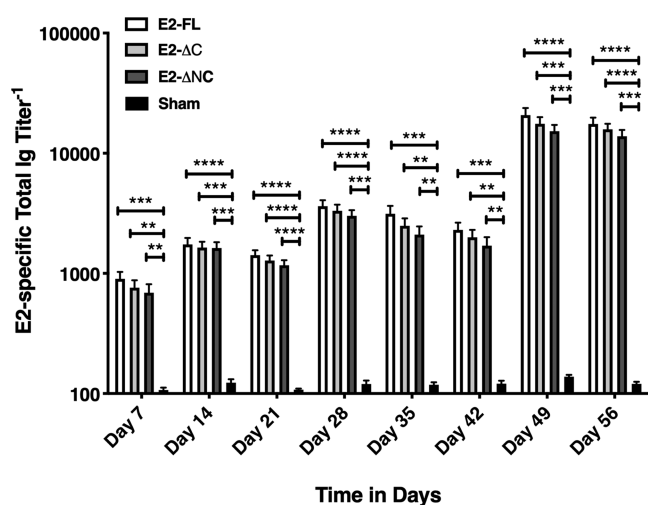


Figure 10. CHIKV E2-specific total Ig titers in different groups of BALB/c mice immunized on day 0, day 14, and day 42 with E2-FL (open bars), E2- Δ C (gray bars), and E2- Δ NC (dark gray) antigens and sham controls (black bars). Antibody titers are shown on the y-axis, and time in days is indicated on the x-axis. All serum samples were tested in duplicate. Bars represent mean \pm scanning electron microscopy (SEM) ($n = 5$). P values are determined using ordinary one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. Differences between groups are indicated by bars and symbols: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

FL, E2- Δ C, and E2- Δ NC proteins for the presence or absence of seven B-cell epitopes identified by Chua et al.,²⁴ we found the presence of all seven epitopes in the CHIKV E2-FL and E2- Δ C protein fragments (Table 3). However, the E2- Δ NC protein fragment showed the presence of only six epitopes, wherein the N-terminal epitope (16–30 aa) was found absent (shown in blue, Figure 11). On comparing the sequences of CHIKV E2-FL, E2- Δ C, and E2- Δ NC proteins for the presence or absence of 17 B-cell epitopes identified by Verma et al.,²⁹ we found all 17 epitopes to be present in the CHIKV E2-FL and E2- Δ C protein fragments (Table 3). However, the E2- Δ NC protein fragment showed the presence of only 16 epitopes, wherein the N-terminal epitope (18–32 aa) was found absent (shown in blue, Figure 11).

In the second approach, we performed *in silico* B-cell epitope analysis for CHIKV E2-FL, E2- Δ C, and E2- Δ NC proteins used in the study using two distinct methods, including ABCpred (https://webs.iitd.edu.in/raghava/abcpred/ABC_submission.html) and BCEpred (https://webs.iitd.edu.in/raghava/bcepred/bcepred_submission.html) servers using their default threshold values. Using the ABCpred server, we

identified varying numbers of potential B-cell epitopes in CHIKV E2-FL ($n = 41$), CHIKV E2- Δ C ($n = 36$), and CHIKV E2- Δ NC ($n = 33$) using a window size of 16-mers at 0.51 threshold value. The ABCpred online server predicted four potential B-cell epitopes in the extra C-terminal region of the CHIKV E2-FL protein, which were absent in both the E2- Δ C and E2- Δ NC proteins (shown in red, Figure 11) (Table 4). From the BCEpred server, the potential B-cell epitopes were predicted as follows: CHIKV E2-FL ($n = 15$), CHIKV E2- Δ C ($n = 12$), and CHIKV E2- Δ NC ($n = 11$). According to the BCEpred server (based on accessibility), three extra potential B-cell epitopes were identified in the extra C-terminal region of the CHIKV E2-FL protein at 1.9 threshold value (shown in red, Figure 11) (Table 5). These three epitopes overlapped with the four unique epitopes found in the extra C-terminal region of CHIKV E2-FL as per ABCpred server results.

2.9.2. T-Cell Epitope Analysis. The use of the CHIKV E2-FL protein is expected to improve the efficacy and therapeutic potential of the subunit vaccine against chikungunya infection, as compared to vaccine formulations using truncated fragments of the CHIKV E2 protein, which lack the C-terminal epitopes. To test whether the CHIKV E2-FL protein fares better than the truncated fragments in terms of immunogenic epitopes, we investigated if the extra C-terminal region of the CHIKV E2-FL protein contains immunogenic peptides or better epitopes, which are not present in the truncated CHIKV E2- Δ C and E2- Δ NC fragments. For identifying these T-cell epitopes, we performed *in silico* analysis of the extra C-terminal region of the CHIKV E2-FL protein using T-cell epitope prediction servers available online, which calculate the binding affinity and antigenicity by generating small peptide sequences from the submitted sequence, on the basis of predicted scores above a certain threshold value. Since MHC binding is a prerequisite for T-cell-based immune responses against antigenic peptides, we used this as a parameter for identifying the potential antigenic peptides in the extra region of the CHIKV E2-FL protein.

For identifying human MHC class I binding T-cell epitopes present in the extra region of the CHIKV E2-FL protein, we used the Immune Epitope Database and the Analysis Resource (IEDB) T-cell epitope prediction server, wherein the CHIKV E2-FL protein C-terminal sequence spanning 366–423 aa (58 aa) was submitted as the query sequence. The IEDB-recommended NetMHCpan EL 4.1 (<http://tools.iiedb.org/mhci/>, version 2020.09) method was used to predict the MHC class I binding affinity of T-cell epitopes (of 8–14 mer length) for 77 human MHC class I alleles present in the IEDB server. The server predicts the binding affinity of each peptide with multiple MHC alleles, which resulted in the total number of

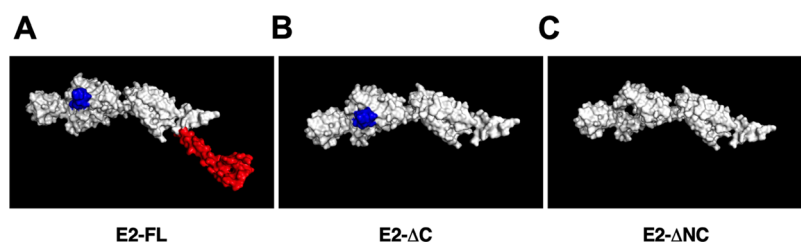


Figure 11. Tertiary structures of (A) CHIKV E2-FL, (B) E2- Δ C, and (C) E2- Δ NC proteins. The N-terminal B-cell epitopes (1–19 and 16–32 aa) present only in E2-FL and E2- Δ C proteins are shown in blue. The extra C-terminal region (366–423 aa), present only in the E2-FL protein (containing four additional B-cell epitopes), is shown in red.

Table 3. B-Cell Epitopes (as per References Cited) Present in CHIKV E2 Full-Length and Truncated Proteins Used in the Study[¶]

SN	E2-FL ^a	Position	E2-ΔC ^a	Position	E2-ΔNC ^a	Position
1	STKDNFNVYKATRPYLAHC^{***}	1-19	STKDNFNVYKATRPYLAHC	1-19	Absent	-
2	TDGTLKIQVSLQIGIKTDDSHDWTCLRYMDNHMPA DAERAGL [†]	42-83	TDGTLKIQVSLQIGIKTDDSHDWTCLRYM DNHMPADAERAGL	42-83		42-83
3	HHDPVPIGREKFHSRPOHGKELPCST [†]	130-155	HHDPVPIGREKFHSRPOHGKELPCST	130-155		130-155
4	ATTEEIEVHMPPDTPDRT [†]	162-179	ATTEEIEVHMPPDTPDRT	162-179		162-179
5	GNVKITVNGQTVRYKNC [†]	186-203	GNVKITVNGQTVRYKNC	186-203		186-203
6	LTTTDRKVINNCKVDQCHAAVTNHKKW [†]	210-235	LTTTDRKVINNCKVDQCHAAVTNHKKW	210-235		210-235
7	HAAVTNHKKWQYNSPLVPRNAELGDRKGIHIPP LANVTCRVPKARNPTVTVYKGNQV^{†*}	226-282	HAAVTNHKKWQYNSPLVPRNAELGDRKGIHIPP LANVTCRVPKARNPTVTVYKGNQV	226-282		226-282
9	PTVTVYKGNQVIMLLYPDHPHTLLSYRN^{††}	274-299	PTVTVYKGNQVIMLLYPDHPHTLLSYRN	274-299		274-299
10	RNMGEFPNYQEEWVMHKKVEVLTVPTEGLEVTWGN NEPYKYWPQLSNGT^{††}	298-347	RNMGEFPNYQEEWVMHKKVEVLTVPTEGLE VWGNNEPYKYWPQLSNGT	298-347		298-347
11	LLSMVGMAGMCMCARRRCITPYELTPGATVPFL^{††}	378-411	Absent	-	Absent	-
SN	E2-FL ^b	Position	E2-ΔC ^b	Position	E2-ΔNC ^b	Position
1	LAHCPDCGEGHSCHS	16-30	LAHCPDCGEGHSCHS	16-30	Absent	-
2	ADAERAGLFV	76-85	ADAERAGLFV	76-85		76-85
3	THPFHDDPPV	126-134	THPFHDDPPV	126-134		126-134
4	EIEVHMPPDT	166-174	EIEVHMPPDT	166-174		166-174
5	GEEPNYQEEW	301-310	GEEPNYQEEW	301-310		301-310
6	VPTGLEVTW	321-330	VPTGLEVTW	321-330		321-330
7	GNNEPYKYWP	331-340	GNNEPYKYWP	331-340		331-340
SN	E2-FL ^c	Position	E2-ΔC ^c	Position	E2-ΔNC ^c	Position
1	HCPDCGEGHSCHSPV	18-32	HCPDCGEGHSCHSPV	18-32	Absent	-
2	RIRNEATDGTLLKI	36-48	RIRNEATDGTLLKI	36-48		36-48
3	IKTDDSHDWTCLRY	56-69	IKTDDSHDWTCLRY	56-69		56-69
4	KLRVMDNHIPADAGRA	66-81	KLRVMDNHIPADAGRA	66-81		66-81
5	GLFVRTSAPCTITGTM	82-97	GLFVRTSAPCTITGTM	82-97		82-97
6	GFTDSKISHSCTHPFHHD	114-132	GFTDSKISHSCTHPFHHD	114-132		114-132
7	PVIGREKFHSRPOHGKELPC	134-153	PVIGREKFHSRPOHGKELPC	134-153		134-153
8	TYVQSTAAATTEIEVHMPP	155-173	TYVQSTAAATTEIEVHMPP	155-173		155-173
9	DTPDRTLMSQOSGNVKIT	174-191	DTPDRTLMSQOSGNVKIT	174-191		174-191
10	VNGRTVRYKNCNGGSGNEG	192-209	VNGRTVRYKNCNGGSGNEG	192-209		192-209
11	DKVINNCKVDQCHAAVTNHK	214-233	DKVINNCKVDQCHAAVTNHK	214-233		214-233
12	NSPLVPRNAELGDRKKG	238-254	NSPLVPRNAELGDRKKG	238-254		238-254
13	RVPKARNPTVTVYKGNQ	267-282	RVPKARNPTVTVYKGNQ	267-282		267-282
14	NMGEFPNYQEEWVMHK	299-314	NMGEFPNYQEEWVMHK	299-314		299-314
15	VPTGLEVTWGNNEPY	321-336	VPTGLEVTWGNNEPY	321-336		321-336
16	LSTNGTAHGHPHE	342-354	LSTNGTAHGHPHE	342-354		342-354
17	HEILYYEYLYP	353-364	HEILYYEYLYP	353-364		353-364

^aE2 epitopes identified as per ref 32. ^bE2 epitopes identified as per ref 24. ^cE2 epitopes identified as per ref 70. [†]E2 epitopes identified in humans as per ref 22. ^{**}E2 epitopes identified in mouse as per ref 25. ^{††}E2 epitopes identified in macaque as per ref 33. [¶]E2 epitope residues conserved in humans and mouse are indicated in bold. The N-terminal epitope (1-19 aa and 16-32 aa) is listed in blue, present in CHIKV E2-FL and E2-ΔC proteins. The C-terminal epitope (378-411 aa) present only in the CHIKV E2-FL protein is shown in red.

peptides being more than the total overlapping peptides possible in the extra region of the protein. We found a total of 2673 CD8 T-cell potential peptides binding to 27 human MHC I alleles. As per published reports,^{35,36} T-cell epitopes with an MHC class I binding score > 0.75 are considered good binders. We identified two potential T-cell epitopes (ATVPFLLSL: 0.87 and YELTPGATV: 0.85) in the CHIKV E2-FL extra C-terminal region binding exclusively to HLA-A*02:06 and HLA-B*40:01 MHC class I alleles, respectively, whose score value > 0.75 (Table 6). These two potential epitopes were absent in both the truncated (E2-ΔC and E2-ΔNC) fragments.

Further, we analyzed the MHC class II binding T-cell epitopes present in the extra region of the CHIKV E2-FL protein using the default IEDB-recommended 2.22 method (<http://tools.iedb.org/mhcii/>). The human MHC class II binding T-cell epitopes (of default 15-mer length) were analyzed with respect to 1190 human MHC II alleles present in the IEDB server. The server predicts the binding affinity of each peptide with multiple MHC alleles, resulting in the total number of peptides being more than the total overlapping peptides possible in the extra region of the protein. We obtained a total of 1188 CD4 T-cell peptides in the selected CHIKV E2 C-terminal sequence, binding to 28 human MHC II alleles. As per the literature, the standard peptide affinity measurements were followed, viz., IC₅₀ values < 50 nM considered as higher affinity, IC₅₀ values < 500 nM considered

as intermediate affinity, and IC₅₀ < 5000 nM indicated a lower affinity of the epitopes.^{35,37} By assigning a cutoff value of IC₅₀ < 500 nM, we identified a total of 253 peptides with good binding affinities, out of which 37 were unique T-cell peptides (listed in Table 6 along with IC₅₀ values), which showed good binding to 19 human MHC class II alleles.

Although peptide binding to MHC class I and MHC class II molecules is necessary for T-cell responses, it does not guarantee its immunogenic nature. To investigate how many of the good binding peptides are immunogenic, we used the IEDB CD4 T-cell immunogenicity prediction program (<http://tools.iedb.org/CD4episcore/>) to filter for immunogenic peptides. We found a total of nine immunogenic peptides in the CHIKV E2-FL protein, six in CHIKV E2-ΔC, and four T-cell-specific immunogenic peptides in the CHIKV E2-ΔNC fragment. On keeping a cutoff score > 90, we found two highly immunogenic peptides (MTVVVVSVATFILLS: 366-380 aa position; immunogenicity score: 97.67; and FILLSMVG-MAAGMCM: 376-390 aa position; immunogenicity score: 90.43) in the extra C-terminal region of CHIKV E2-FL (Table 7). Interestingly, the highest predicted immunogenic peptide sequence (MTVVVVSVATFILLS; score 97.67) among all three CHIKV E2 fragments analyzed was located in the extra region of the full-length protein and is absent in the two truncated E2 fragments.

Since we wanted to check what fraction of human MHC alleles can potentially present these good binding peptides

Table 4. List of Predicted B-Cell Epitopes in CHIKV E2 Protein Fragments Using the ABCpred Server (Epitope Threshold = 0.51)

SN	E2-FL	Position	E2-AC	Position	E2-ANC	Position
1	FNVYKATRPYLAHCPD	6-21	FNVYKATRPYLAHCPD	6-21	Absent	-
2	LAHCPDCGEGHSCHSP	16-31	LAHCPDCGEGHSCHSP	16-31	Absent	-
3	CHSPVALERIRNEATD	28-43	CHSPVALERIRNEATD	28-43	Absent	-
4	RIRNEATDGTLLKIQVS	36-51	RIRNEATDGTLLKIQVS	36-51	RIRNEATDGTLLKIQVS	36-51
5	QIGIKTDDSHDWTCLR	53-68	QIGIKTDDSHDWTCLR	53-68	QIGIKTDDSHDWTCLR	53-68
6	SHDWTCLRMDNHMPA	61-76	SHDWTCLRMDNHMPA	61-76	SHDWTCLRMDNHMPA	61-76
7	AERAGLFVRTSAPCTI	78-93	AERAGLFVRTSAPCTI	78-93	AERAGLFVRTSAPCTI	78-93
8	CTITGTMGHFILARCP	91-106	CTITGTMGHFILARCP	91-106	CTITGTMGHFILARCP	91-106
9	GHFILARCPKGETLTV	98-113	GHFILARCPKGETLTV	98-113	GHFILARCPKGETLTV	98-113
10	TLTVGFTDSRKISHSC	110-125	TLTVGFTDSRKISHSC	110-125	TLTVGFTDSRKISHSC	110-125
11	SRKISHSCTHPFHHPD	118-133	SRKISHSCTHPFHHPD	118-133	SRKISHSCTHPFHHPD	118-133
12	CTHPFHHPDPPVIGREK	125-140	CTHPFHHPDPPVIGREK	125-140	CTHPFHHPDPPVIGREK	125-140
13	DPPVIGREKFHSRPOH	132-147	DPPVIGREKFHSRPOH	132-147	DPPVIGREKFHSRPOH	132-147
14	REKFHSRPOHGKELPC	138-153	REKFHSRPOHGKELPC	138-153	REKFHSRPOHGKELPC	138-153
15	ELPCSTYVQSTAATTE	150-165	ELPCSTYVQSTAATTE	150-165	ELPCSTYVQSTAATTE	150-165
16	AATTEIEVHMPPDTP	161-176	AATTEIEVHMPPDTP	161-176	AATTEIEVHMPPDTP	161-176
17	IEVHMPPDTPDRTLMS	167-182	IEVHMPPDTPDRTLMS	167-182	IEVHMPPDTPDRTLMS	167-182
18	PDTPDRTLMSQQSGNV	173-188	PDTPDRTLMSQQSGNV	173-188	PDTPDRTLMSQQSGNV	173-188
19	MSQQSGNVKITVNGQT	181-196	MSQQSGNVKITVNGQT	181-196	MSQQSGNVKITVNGQT	181-196
20	VRYKCNCGGSNEGLTT	197-212	VRYKCNCGGSNEGLTT	197-212	VRYKCNCGGSNEGLTT	197-212
21	GGSGNEGLTTDKVINN	204-219	GGSGNEGLTTDKVINN	204-219	GGSGNEGLTTDKVINN	204-219
22	DKVINNCKVDQCHAAV	214-229	DKVINNCKVDQCHAAV	214-229	DKVINNCKVDQCHAAV	214-229
23	VDQCHAAVTNHHKKWQY	222-237	VDQCHAAVTNHHKKWQY	222-237	VDQCHAAVTNHHKKWQY	222-237
24	AVTNHHKKWQYNSPLVP	228-243	AVTNHHKKWQYNSPLVP	228-243	AVTNHHKKWQYNSPLVP	228-243
25	KWQYNSPLVPRNAELG	234-249	KWQYNSPLVPRNAELG	234-249	KWQYNSPLVPRNAELG	234-249
26	PRNAELGDRKGIHIP	243-258	PRNAELGDRKGIHIP	243-258	PRNAELGDRKGIHIP	243-258
27	KIHIFPPLANVTCRVP	254-269	KIHIFPPLANVTCRVP	254-269	KIHIFPPLANVTCRVP	254-269
28	NVTCRVPKARNPTVTY	263-278	NVTCRVPKARNPTVTY	263-278	NVTCRVPKARNPTVTY	263-278
29	PKARNPTVTYGKNQVI	269-284	PKARNPTVTYGKNQVI	269-284	PKARNPTVTYGKNQVI	269-284
30	HTLLSYRNMGEPEPNY	291-306	HTLLSYRNMGEPEPNY	291-306	HTLLSYRNMGEPEPNY	291-306
31	MLLYPDHPTLLSYRNM	285-300	MLLYPDHPTLLSYRNM	285-300	MLLYPDHPTLLSYRNM	285-300
32	EEWVMHKKVVLTVPT	308-323	EEWVMHKKVVLTVPT	308-323	EEWVMHKKVVLTVPT	308-323
33	LTVPTGLEVTWGNNE	319-334	LTVPTGLEVTWGNNE	319-334	LTVPTGLEVTWGNNE	319-334
34	GLEVTWGNNEPKYWP	325-340	GLEVTWGNNEPKYWP	325-340	GLEVTWGNNEPKYWP	325-340
35	GNNEPKYWPQLSTNG	331-346	GNNEPKYWPQLSTNG	331-346	GNNEPKYWPQLSTNG	331-346
36	HGHPHEIILYYEYLYP	349-364	HGHPHEIILYYEYLYP	349-364	HGHPHEIILYYEYLYP	349-364
37	LYYYEYLYPTMTVVVVS	357-372	LYYYEYLYPTMTVVVVS	357-365	LYYYEYLYPTMTVVVVS	357-365
38	TFILLSMVGMAAGMCM	375-390	Absent	-	Absent	-
39	MAAGMCMARRRCITP	384-399	Absent	-	Absent	-
40	RRRCITPYELTPGATV	393-408	Absent	-	Absent	-
41	ELTPGATVPFLLSLIC	401-416	Absent	-	Absent	-

Table 5. List of Predicted B-Cell Epitopes in CHIKV E2 Protein Fragments Using the BCEpred Server (Epitope Threshold = 1.9)

S.N	E2-FL Peptide Sequence	Position	E2-AC Peptide Sequence	Position	E2-ANC Peptide Sequence	Position
1	HSCHSPV	26-32	HSCHSPV	26-32	Absent	-
2	TLKIQVSLQIGI	45-56	TLKIQVSLQIGI	45-56	TLKIQVSLQIGI	45-56
3	ISHSCTHPFHHPDPPVI	121-136	ISHSCTHPFHHPDPPVI	121-136	ISHSCTHPFHHPDPPVI	121-136
4	ELPCSTYVQVS	150-159	ELPCSTYVQVS	150-159	ELPCSTYVQVS	150-159
5	QTVRYKCN	195-203	QTVRYKCN	195-203	QTVRYKCN	195-203
6	VINNCKVDQCH	216-226	VINNCKVDQCH	216-226	VINNCKVDQCH	216-226
7	QYNSPLVP	236-243	QYNSPLVP	236-243	QYNSPLVP	236-243
8	IHIPFPL	255-261	IHIPFPL	255-261	IHIPFPL	255-261
9	NVTCRVPK	263-270	NVTCRVPK	263-270	NVTCRVPK	263-270
10	NQVIMLLYPDHPHTLLSY	281-297	NQVIMLLYPDHPHTLLSY	281-297	NQVIMLLYPDHPHTLLSY	281-297
11	HKKEVVLTVPTE	313-324	HKKEVVLTVPTE	313-324	HKKEVVLTVPTE	313-324
12	HPHEIILYYEYLYPTMTVVVVSVA	351-374	HPHEIILYYEYLYP	351-364	HPHEIILYYEYLYP	351-364
13	FILLSMVG	376-383	Absent	-	Absent	-
14	CITPYEL	396-402	Absent	-	Absent	-
15	TVPFLLSLICIRT	407-420	Absent	-	Absent	-

during CHIKV infection, the allele cumulative percentage frequency graphs were plotted for the selected two highly immunogenic epitopes in the C-terminal region of CHIKV E2-FL, based upon $IC_{50} < 500$ nM (Figure 12). The alleles that exhibit good binding affinities with these two peptides are listed in Table 8. Human MHC genes are highly polymorphic, which means that many different alleles exist in different individuals inside a population. T-cells recognize a complex between a specific MHC molecule and an antigenic epitope, playing an important role in immune function. Useful antigenic

epitopes bind to the large spectrum of MHC alleles. Therefore, in the design and the development of vaccines, selecting epitopes binding to multiple MHC alleles will increase population coverage. If an epitope binds to all alleles of the MHC class, then it would be able to generate effective immune response in the entire population. However, there may not be a single antigenic peptide that binds to all MHC alleles in a population. We investigated the coverage of selected immunogenic epitopes present in the extra C-terminal region of the CHIKV E2-FL protein with multiple MHC class II

Table 6. List of MHC Class II and MHC Class II T-Cell Epitopes in the Extra C-Terminal Region of the CHIKV E2-FL Protein Predicted Using the IEDB Online Server Along with Their Alleles

SN	peptide	position (aa)	identity of human MHC II alleles
1	AAGMCMCARRRCITP	385–399	HLA-DRB5*01:01, HLA-DRB1*11:01
2	AGMCMCARRRCITPY	386–400	HLA-DRB1*11:01
3	ATFLLSMVGMAGM	374–388	HLA-DRB1*01:01, HLA-DRB1*04:01, HLA-DQA1*05:01/DQB1*03:01, HLA-DRB1*11:01, HLA-DRB1*04:05, HLA-DRB4*01:01, HLA-DQA1*01:02/DQB1*06:02, HLA-DRB1*15:01, HLA-DRB1*09:01, HLA-DRB1*03:01, HLA-DRB1*03:01, HLA-DPAI*03:01/DPB1*04:02, HLA-DRB1*07:01
4	ATVPFLLSLICIRT	406–420	HLA-DRB1*01:01, HLA-DRB1*07:01, HLA-DPAI*03:01/DPB1*04:02, HLA-DRB1*04:05, HLA-DPAI*01:03/DPB1*02:01, HLA-DRB1*15:01
5	CITPYELTPGATVPF	396–410	HLA-DRB1*01:01, HLA-DRB1*07:01, HLA-DRB1*09:01, HLA-DQA1*05:01/DQB1*03:01, HLA-DRB1*11:01
6	ELTPGATVPFLLSLI	401–415	HLA-DPAI*03:01/DPB1*04:02, HLA-DPAI*02:01/DPB1*01:01, HLA-DQA1*05:01/DQB1*03:01, HLA-DPAI*01:03/DPB1*02:01
7	FILLSMVGMAGMCM	376–390	HLA-DRB1*01:01, HLA-DQA1*05:01/DQB1*03:01, HLA-DRB1*04:01, HLA-DQA1*01:02/DQB1*06:02, HLA-DRB1*04:05, HLA-DRB1*09:01, HLA-DRB4*01:01, HLA-DRB1*15:01, HLA-DRB1*07:01
8	GATVPFLLSLICIR	405–419	HLA-DRB1*01:01, HLA-DPAI*03:01/DPB1*04:02, HLA-DRB1*07:01, HLA-DPAI*01:03/DPB1*02:01
9	GMAAGMCMCARRRCI	383–397	HLA-DRB5*01:01, HLA-DRB1*11:01
10	ILLSMVGMAGMCMC	377–391	HLA-DRB1*01:01, HLA-DQA1*05:01/DQB1*03:01, HLA-DQA1*01:02/DQB1*06:02, HLA-DRB1*04:01
11	ITPYELTPGATVPFL	397–411	HLA-DRB1*01:01, HLA-DRB1*07:01, HLA-DRB1*09:01, HLA-DQA1*05:01/DQB1*03:01, HLA-DRB1*11:01
12	LLSMVGMAGMCMCA	378–392	HLA-DRB1*01:01, HLA-DQA1*05:01/DQB1*03:01, HLA-DQA1*01:02/DQB1*06:02, HLA-DRB1*04:01
13	LSMVGMAGMCMCAR	379–393	HLA-DRB1*01:01, HLA-DQA1*05:01/DQB1*03:01, HLA-DQA1*01:02/DQB1*06:02
14	LTPGATVPFLLSLIC	402–416	HLA-DPAI*03:01/DPB1*04:02, HLA-DPAI*02:01/DPB1*01:01, HLA-DPAI*01:03/DPB1*02:01, HLA-DRB1*07:01
15	MAAGMCMCARRRCIT	384–398	HLA-DRB5*01:01, HLA-DRB1*11:01
16	MTVVVSVATFILLS	366–380	HLA-DPAI*02:01/DPB1*01:01, HLA-DRB1*07:01, HLA-DPAI*01:03/DPB1*02:01, HLA-DRB1*01:01, HLA-DPAI*03:01/DPB1*04:02, HLA-DQA1*01:02/DQB1*06:02, HLA-DRB5*01:01, HLA-DRB1*15:01, HLA-DRB1*09:01, HLA-DRB1*08:02, HLA-DQA1*05:01/DQB1*03:01, HLA-DPAI*02:01/DPB1*05:01, HLA-DRB1*03:01, HLA-DRB1*04:05, HLA-DRB4*01:01
17	MVGMAGMCMCARRR	381–395	HLA-DQA1*01:02/DQB1*06:02, HLA-DRB1*01:01, HLA-DRB5*01:01, HLA-DQA1*05:01/DQB1*03:01
18	PFLLSLICIRTA	409–423	HLA-DRB1*01:01, HLA-DRB1*11:01, HLA-DRB1*04:01, HLA-DRB1*07:01, HLA-DRB4*01:01
19	PGATVPFLLSLICCI	404–418	HLA-DPAI*03:01/DPB1*04:02, HLA-DRB1*01:01, HLA-DRB1*07:01, HLA-DPAI*01:03/DPB1*02:01, HLA-DPAI*02:01/DPB1*01:01
20	PYELTPGATVPFLLS	399–413	HLA-DRB1*01:01, HLA-DRB1*07:01, HLA-DQA1*05:01/DQB1*03:01, HLA-DRB1*09:01
21	RCITPYELTPGATVP	395–409	HLA-DRB1*01:01, HLA-DRB1*07:01, HLA-DRB1*09:01, HLA-DQA1*05:01/DQB1*03:01, HLA-DRB1*11:01
22	RRCITPYELTPGATV	394–408	HLA-DRB1*01:01, HLA-DRB1*07:01, HLA-DRB1*09:01, HLA-DRB1*11:01

Table 6. continued

SN	peptide	position (aa)	identity of human MHC II alleles
23	SMVGMAGMCMCARR	380–394	HLA-DRB1*01:01, HLA-DQA1*01:02/DQB1*06:02, HLA-DQA1*05:01/DQB1*03:01
24	SVATFILLSMVGMAA	372–386	HLA-DRB1*01:01, HLA-DRB1*04:01, HLA-DRB1*11:01, HLA-DRB1*15:01, HLA-DRB4*01:01, HLA-DPAI*03:01/DPB1*04:02, HLA-DQAI*01:02/DQB1*06:02, HLA-DQAI*05:01/DQB1*03:01, HLA-DRB1*09:01, HLA-DRB1*03:01, HLA-DRB5*01:01, HLA-DRB1*07:01, HLA-DRB1*12:01
25	TFILLSMVGMAAGMC	375–389	HLA-DRB1*01:01, HLA-DQAI*05:01/DQB1*03:01, HLA-DRB1*04:01, HLA-DQAI*01:02/DQB1*06:02, HLA-DRB1*04:05, HLA-DRB1*11:01, HLA-DRB1*03:01, HLA-DRB1*09:01, HLA-DRB1*15:01, HLA-DRB4*01:01
26	TPGATVPFLLSICC	403–417	HLA-DPAI*03:01/DPB1*04:02, HLA-DPAI*02:01/DPB1*01:01, HLA-DPAI*01:03/DPB1*02:01
27	TPYELTPGATVPFLL	398–412	HLA-DRB1*01:01, HLA-DRB1*07:01, HLA-DRB1*09:01, HLA-DQAI*05:01/DQB1*03:01, HLA-DRB1*11:01
28	TVPFLLSICCIRTA	407–421	HLA-DRB1*01:01, HLA-DRB1*07:01, HLA-DRB1*04:05, HLA-DPAI*03:01/DPB1*04:02, HLA-DPAI*01:03/DPB1*02:01
29	TVVVSVATFILLSM	367–381	HLA-DPAI*02:01/DPB1*01:01, HLA-DRB1*07:01, HLA-DPAI*01:03/DPB1*02:01, HLA-DPAI*03:01/DPB1*04:02, HLA-DRB1*01:01, HLA-DRB1*13:02, HLA-DQAI*01:02/DQB1*06:02, HLA-DRB1*15:01, HLA-DRB5*01:01, HLA-DRB1*09:01, HLA-DRB1*02:01/DPB1*05:01, HLA-DRB1*04:05, HLA-DQAI*05:01/DQB1*03:01, HLA-DRB1*08:02, HLA-DRB1*03:01, HLA-DRB1*04:01, HLA-DRB4*01:01
30	VATFILLSMVGMAAG	373–387	HLA-DRB1*01:01, HLA-DRB1*04:01, HLA-DRB1*11:01, HLA-DRB1*04:05, HLA-DRB1*15:01, HLA-DRB4*01:01, HLA-DQAI*05:01/DQB1*03:01, HLA-DPAI*03:01/DPB1*04:02, HLA-DQAI*01:02/DQB1*06:02, HLA-DRB1*09:01, HLA-DRB5*01:01, HLA-DRB1*03:01, HLA-DRB1*12:01, HLA-DRB1*07:01, HLA-DPAI*02:01/DPB1*01:01
31	VGMAAGMCMCARRRC	382–396	HLA-DQAI*01:02/DQB1*06:02, HLA-DRB5*01:01, HLA-DQAI*05:01/DQB1*03:01, HLA-DRB1*01:01
32	VPFLLSICCIRTAK	408–422	HLA-DRB1*01:01, HLA-DRB1*07:01, HLA-DRB1*11:01, HLA-DRB1*04:05, HLA-DPAI*03:01/DPB1*04:02
33	VSVATFILLSMVGMA	371–385	HLA-DRB1*01:01, HLA-DPAI*02:01/DPB1*01:01, HLA-DPAI*03:01/DPB1*04:02, HLA-DPAI*01:03/DPB1*02:01, HLA-DRB1*11:01, HLA-DRB4*01:01, HLA-DRB1*15:01, HLA-DQAI*01:02/DQB1*06:02, HLA-DRB1*04:01, HLA-DRB1*04:05, HLA-DRB1*03:01, HLA-DRB5*01:01, HLA-DQAI*05:01/DQB1*03:01, HLA-DRB1*12:01, HLA-DRB1*09:01
34	VVSVATFILLSMVGGM	370–384	HLA-DRB1*01:01, HLA-DPAI*02:01/DPB1*01:01, HLA-DPAI*03:01/DPB1*04:02, HLA-DPAI*01:03/DPB1*02:01, HLA-DQAI*01:02/DQB1*06:02, HLA-DRB1*07:01, HLA-DRB4*01:01, HLA-DRB1*15:01, HLA-DRB1*04:01, HLA-DRB1*04:05, HLA-DRB5*01:01
35	VVVSATFILLSMVG	369–383	HLA-DPAI*02:01/DPB1*01:01, HLA-DPAI*01:03/DPB1*02:01, HLA-DPAI*03:01/DPB1*04:02, HLA-DRB1*07:01, HLA-DQAI*01:02/DQB1*06:02, HLA-DRB1*01:01, HLA-DRB1*15:01, HLA-DRB1*13:02, HLA-DPAI*02:01/DPB1*05:01, HLA-DRB1*11:01, HLA-DRB1*04:05
36	VVVSVATFILLSMV	368–382	HLA-DPAI*02:01/DPB1*01:01, HLA-DPAI*01:03/DPB1*02:01, HLA-DRB1*07:01, HLA-DPAI*03:01/DPB1*04:02, HLA-DRB1*01:01, HLA-DQAI*01:02/DQB1*06:02, HLA-DRB1*15:01, HLA-DRB4*01:01, HLA-DRB1*13:02, HLA-DPAI*02:01/DPB1*05:01, HLA-DRB1*08:02, HLA-DRB1*04:05, HLA-DRB1*04:01
37	YELTPGATVPFLLSL	400–414	HLA-DRB1*01:01, HLA-DRB1*07:01, HLA-DPAI*03:01/DPB1*04:02, HLA-DQAI*05:01/DQB1*03:01, HLA-DRB1*09:01, HLA-DPAI*01:03/DPB1*02:01, HLA-DPAI*02:01/DPB1*01:01

Table 7. List of Immunogenicity Score for Each MHC Class II Alleles to the Predicted CHIKV E2-FL, E2-ΔC, and E2-ΔNC Proteins

SN	CHIKV E2-FL	Position	Imm.Score	CHIKV E2-ΔC	Position	Imm.Score	CHIKV E2-ΔNC	Position	Imm.Score
1	STKDNFNVYKATRPY	1-15	77.0213	STKDNFNVYKATRPY	1-15	77.0213	Absent	-	-
2	FNVYKATRPYLAHCP	6-20	83.2753	FNVYKATRPYLAHCP	6-20	83.2753	Absent	-	-
3	ATDGTSLKIQVSLQIG	41-55	92.0637	ATDGTSLKIQVSLQIG	41-55	92.0637	EATDGTSLKIQVSLQI	40-54	92.2718
4	LKIQVSLQIGIKTDD	46-60	92.1315	LKIQVSLQIGIKTDD	46-60	92.1315	TLKIQVSLQIGIKTD	45-59	91.639
5	GNVKITVNGQTVRYK	186-200	88.7022	GNVKITVNGQTVRYK	186-200	88.7022	SGNVKITVNGQTVRY	185-199	91.436
6	NQVIMLLYPDHPDLL	281-295	81.086	NQVIMLLYPDHPDLL	281-295	81.086	MLLYPDHPDLLSYRN	285-299	96.8817
7	ILYYYELYPTMTVVV	356-370	83.0174	Absent	-	-	Absent	-	-
8	MTVVVVSVATFILLS	366-380	97.6789	Absent	-	-	Absent	-	-
9	FILLSMVGMAAGMCM	376-390	90.4386	Absent	-	-	Absent	-	-

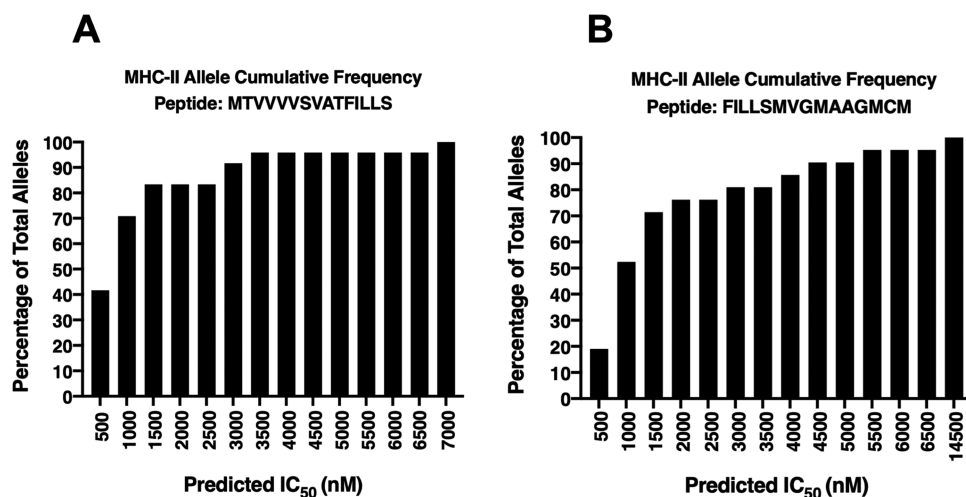


Figure 12. Graphical representation of the predicted binding affinity of potential T-cell epitopes (A) MTVVVVSVATFILLS and (B) FILLSMVGMAAGMCM from the extra C-terminal region of the CHIKV E2 protein to MHC class II alleles. The percentage of total MHC class II alleles is depicted on the y-axis, with the predicted affinities (IC_{50}) to MHC class II depicted on the x-axis.

alleles. We calculated the fractions of human MHC class II alleles with different binding affinities to two highly immunogenic T-cell epitopes predicted from the extra C-terminal region of the E2-FL CHIKV protein. From the allele cumulative frequency distribution graphs, we found that the predicted T-cell epitope MTVVVVSVATFILLS (score 97.67) binds to 41% of the class II MHC alleles ($IC_{50} < 500$ nM) (Figure 12A). The other predicted T-cell epitope FILLSMVGMAAGMCM (score 90.43) showed 19% binding to MHC class II alleles (Figure 12B). This implies that these two peptide epitopes, which are present only in the CHIKV E2-FL protein, are capable of mounting a diverse immune response in a large fraction of the population. It is likely that a vaccine containing such types of peptide epitopes will have a large population coverage and will provide future direction for vaccine design and development against chikungunya infection.

We also performed the analysis for the identification of T-cell epitopes for the extra region of the CHIKV E2-FL protein using the IEDB server for mouse MHC class I and class II alleles. We did not find any antigenic peptide in the extra region of the E2-FL protein whose binding score was >0.75 when the analysis was performed for mouse MHC class I alleles. Similarly, we did not find any antigenic peptide in the extra region of the CHIKV E2-FL protein whose binding affinity (IC_{50}) was less than 500 nM for mouse MHC class II alleles.

3. DISCUSSION

Ongoing research efforts are underway to develop new methodologies for efficient diagnosis, monitoring, and controlling of chikungunya infections. The development of anti-CHIKV subunit vaccines and more efficient serological tests depends on the isolation and purification of surface immunogenic viral antigens, among which the envelope E2 protein is a prime vaccine candidate, successfully evaluated in separate studies.^{38,39} The CHIKV E2 protein is a highly immunogenic viral protein, involved in host–cell interaction and capable of inducing strong antibody response against chikungunya infection.³² Multiple studies have reported cloning, expression, and purification of CHIKV E2 proteins using the prokaryotic expression system.^{19,29,38,40–42} However, almost all of the previously reported studies have described expression and purification of the truncated E2 protein. Additionally, few studies have demonstrated expression and purification of the CHIKV E2 protein using the baculovirus-based insect cell expression system and HEK-293T mammalian cells, which allow post-translational modifications like glycosylation.^{43–45} However, on comparing the performance of the two CHIKV E2 antigens, a recent study found that both the prokaryotically and eukaryotically expressed E2 proteins demonstrated similar performance for indirect ELISA using anti-CHIKV E2 IgG antibodies.⁴⁵ Since recombinant proteins expressed in the prokaryotic system are much simpler, faster, cost-effective, and usually obtained in higher quantities when compared to proteins expressed in the eukaryotic system, we

Table 8. List of MHC Class II Alleles and Predicted IC₅₀ (nM) to Both Peptides: MTVVVVSVATFILLS (366–380 aa) and FILLSMVGMAAGMCM (376–390 aa)

peptide	MTVVVVSVATFILLS (366–380 aa)	predicted IC ₅₀ < 500 nM
S.N	hMHCII Allele	Predicted IC ₅₀ (nM)
1	HLA-DPA1*02:01/DPB1*01:01	16
2	HLA-DRB1*07:01	19
3	HLA-DPA1*01:03/DPB1*02:01	26
4	HLA-DRB1*01:01	44
5	HLA-DPA1*03:01/DPB1*04:02	57
6	HLA-DRB1*13:02	92
7	HLA-DQA1*01:02/DQB1*06:02	164
8	HLA-DRB5*01:01	230
9	HLA-DRB1*15:01	234
10	HLA-DRB1*09:01	245
11	HLA-DRB1*08:02	254
12	HLA-DQA1*05:01/DQB1*03:01	322
13	HLA-DPA1*02:01/DPB1*05:01	351
14	HLA-DRB1*03:01	369
15	HLA-DRB1*04:05	401
16	HLA-DRB4*01:01	455

peptide	FILLSMVGMAAGMCM (376–390 aa)	predicted IC ₅₀ < 500 nM
S.N	hMHCII allele	predicted IC ₅₀ (nM)
1	HLA-DRB1*01:01	9
2	HLA-DQA1*05:01/DQB1*03:01	83
3	HLA-DRB1*04:01	96
4	HLA-DQA1*01:02/DQB1*06:02	232
5	HLA-DRB1*04:05	250
6	HLA-DRB1*11:01	277
7	HLA-DRB1*09:01	310
8	HLA-DRB4*01:01	329
9	HLA-DRB1*15:01	355
10	HLA-DRB1*07:01	431

attempted cloning, expression, and purification of the full-length CHIKV E2 protein in *E. coli* in our study.

In the present study, we describe optimized protocols for cloning, expression, and purification of CHIKV E2-FL and two truncated proteins using bacterial expression vectors and *E. coli* strains. The recombinant E2-FL and truncated proteins were expressed as the His-tagged fusion protein in *E. coli*. For the production of CHIKV E2-FL and truncated proteins, the *E. coli* bacterial cultures were induced during the log phase with 1 mM IPTG at 0.2 OD for 18 h, which resulted in a high protein yield. After centrifugation, the uninduced and induced bacterial pellets were subjected to SDS-PAGE analysis and visualized by Coomassie brilliant blue staining. Clear bands of corresponding molecular weights of 50, 44, and 40 kDa were observed for CHIKV E2-FL, E2-ΔC, and E2-ΔNC proteins, respectively (Figure 4). Solubilization of inclusion bodies in *E. coli* is considered a convenient way to recover target recombinant proteins.^{40,46} Inclusion bodies are protein aggregates that are formed in *E. coli* during the expression of the recombinant protein and can be highly contaminated with bacterial cell wall components. Therefore, for extracting purified proteins, removal of contaminants is essential, which can be performed using guanidine hydrochloride or urea.⁴⁷ Cloning the CHIKV E2 proteins in the pQE-30 Xa vector allowed addition of the N-terminal histidine tag. Since Ni-NTA chromatography is a widely employed strategy for purification of histidine-tagged recombinant proteins, the His-tagged E2 full-length and

truncated fusion proteins were loaded onto the Ni-NTA affinity chromatography column after bacterial lysis. Protein purification was performed under denaturing conditions using urea. The column was washed with a wash buffer containing different concentrations of imidazole (20, 50, and 100 mM). Finally, the column was eluted with 250 mM imidazole elution buffers and the obtained protein fractions were analyzed using SDS-PAGE. We observed clear specific bands for CHIKV E2-FL and truncated proteins with corresponding molecular weights of approximately 50, 44, and 40 kDa for CHIKV E2-FL, E2-ΔC, and E2-ΔNC proteins, respectively (Figure 5). A western blot hybridization was performed using the anti-His antibody, which further confirmed the successful purification of CHIKV E2-FL, E2-ΔC, and E2-ΔNC proteins (Figure 6).

Since the advantages of high-level expression of recombinant proteins as inclusion bodies are offset by heavy losses in yield encountered along the process of refolding in the form of precipitation of misfolded protein, we performed *in vitro* refolding of denatured CHIKV E2-FL and truncated proteins according to a previously published study.⁴⁸ For the improvement of the refolding efficiency of CHIKV E2-FL and E2 truncated proteins, we modified and further optimized the refolding procedure. We observed that the direct refolding method for the refolding of CHIKV E2 denatured proteins led to the formation of protein aggregates and precipitates. On the other hand, by employing a stepwise refolding method, wherein the concentration of urea was decreased gradually in 10 steps (6, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.0156, and 0 M), no aggregates or protein precipitates were found (Figure 7). Our results indicated a refolding yield of ~98% obtained for the three CHIKV E2 proteins used in the study.

We performed structural analysis of the refolded CHIKV E2 proteins using CD and FTIR spectroscopies. Evaluating the CHIKV E2 proteins experimentally by CD measurements indicated that purified CHIKV E2 proteins were present in a refolded conformation. In addition, comparative analysis of the CD spectra obtained for each of the refolded and denatured CHIKV E2 protein samples showed a change in the CD spectra: from a random coil structure in denatured E2 protein samples to a predominantly β-sheet secondary structure in refolded CHIKV E2 protein samples (Figure 8). The CHIKV E2-FL and truncated protein were subjected to FTIR spectroscopy to further affirm the correctly folded state of the *in vitro* refolded CHIKV E2 proteins. The FTIR analysis was performed according to a previously published study.³¹ We found that in the case of each refolded CHIKV E2-FL and E2 truncated protein sample, a sharp and distinct amide band was observed at 1633/1635 cm⁻¹ wavenumbers, which corresponds to β-sheet structures (Figure 9). The results of our CD and FTIR spectroscopy experiments indicate that the purified refolded CHIKV E2-FL and truncated proteins were indeed present in their near-native conformation.¹⁹

It has been reported by many studies that CHIKV envelope proteins could generate protective immune response by producing neutralizing antibodies, making it a promising target for diagnostic and vaccine development against CHIKV.^{39,49,50} An E2-based subunit vaccine was demonstrated to be protective in mice studies,³⁸ and subunit vaccine formulations based on the recombinant envelope E2 protein elicited balanced Th1/Th2 response and virus-neutralizing antibodies in mice.³⁹ In the present study, the immunogenic efficiency of CHIKV E2 proteins was evaluated by immunizing BALB/c mice with E2-FL and truncated proteins along with an alum

adjuvant. We performed an ELISA assay for the analysis of antibody titers produced against the CHIKV E2 antigen in immunized mice (Figure 10). We have reported that recombinant E2 protein immunization induced a high titer of the E2-specific antibody titers in mice. We found that E2-specific antibody titers for the CHIKV E2-FL antigen were consistently higher, while antibody titers obtained after the immunization of E2- Δ C and E2- Δ NC antigens were comparatively less.

Epitope analysis was performed to understand the differences in immunogenicity observed for CHIKV E2-FL, E2- Δ C, and E2- Δ NC antigens used in the study. Various studies have shown that the majority of CHIKV IgG reactive antigenic sequences constitute mainly of continuous linear^{22,25,32} or few discontinuous conformational^{51,52} epitopes mapped on the CHIKV E2 protein. Different studies have identified several epitopes spanning the entire E2 protein.^{34,53} On comparing the presence or absence of identified B-cell epitopes in the three CHIKV E2 protein fragments, we found that the CHIKV E2-FL protein contained the N-terminal epitopes (1–19 and 16–32 aa) along with a few unique epitopes present in the extra C-terminal region (spanning 365–423 aa), compared to the truncated E2 protein fragments. Our findings suggest that the absence of these epitopes may possibly result in reduced immunogenicity in the truncated E2 protein fragments. As opposed to the 11 different regions in the CHIKV E2 protein identified to contain linear epitopes recognized by the anti-CHIKV antibodies during CHIKV infection,²² we found the presence of all 11 regions in the CHIKV E2-FL protein but the absence of the N-terminal (in the E2- Δ NC protein fragment) and C-terminal (in both E2- Δ C and E2- Δ NC protein fragments) regions in truncated E2 proteins. Of note, the N-terminal epitope (STKDNFNVYKATRPYLAH; spanning 1–18 aa), referred to as E2EP3 in earlier studies, is prominently exposed on the viral envelope and has been implicated in the early IgG response to CHIKV in multiple studies.^{32,54,55} However, a separate study demonstrated that the use of the small linear epitope L (spanning 1–12 aa) was not sufficient to induce a protective immune response in mice when used in isolation.⁵⁶

On mapping anti-CHIKV E2 mouse monoclonal antibodies using a total of 84, 15-mer synthetic peptides (each with a 10-mer overlap) covering the E2 protein (1–362 aa), Chua et al. found that mouse B-cell epitopes were distributed at different functional domains of the E2 glycoprotein, namely, at domain A (16–30 and 76–85 aa), junctions of β -ribbons with domains A and B (126–135 aa and 166–175 aa), and domain C (301–310, 321–330, and 331–340 aa).²⁴ In this study, we further identified a few unique B-cell epitopes using two distinct methods ABCpred and BCEpred. Our ABCpred results predicted four potential B-cell epitopes in the extra C-terminal region of the CHIKV E2-FL protein, which were absent in both the truncated CHIKV E2- Δ C and E2- Δ NC proteins (shown in red, Figure 11). Our results using BCEpred predicted three epitopes, which overlapped with the already predicted four unique B-cell epitopes predicted through ABCpred. The importance of epitopes located in the C-terminal region of the CHIKV E2-FL protein has also been reported by Lum et al., who found that the anti-CHIKV antibodies generated in CHIKV-infected mice targeted epitopes located mainly at the C-terminus of the virus E2 glycoprotein (spanning 130–411 aa), along with the N-terminal epitope (1–19 aa).²⁵ Using a bacteriophage Q β

viruslike particle (VLP) platform, Basu et al. reported that epitopes spanning 1–18 and 226–259 aa elicited high-titer antibodies and the C-terminal epitope (spanning 378–411 aa) was the least reactive, possibly due to the truncated E2 protein fragment used in this study.⁵⁷ In an *in silico* B-cell epitope prediction study, the epitope residues at 386 and 388 aa positions were predicted to evoke significant immune response.⁵⁸ Of note, an immuno-informatics study by Hasan et al. reported the peptide region spanning 9 aa from 397 to 405 aa as the most potential B-cell epitope, along with epitopes identified in the N-terminal region (1–29 aa).⁵⁹ Rodriguez et al. also predicted B-cell epitopes located in the N-terminal (spanning 14–22 and 27–35 aa) and C-terminal (spanning 367–389 aa) regions, suggesting that the presence of these amino acids in the CHIKV E2-FL protein may be contributing in its increased immunogenicity.⁶⁰

Since T-cell epitope content is one of the factors that contributes to protein antigenicity, we performed *in silico* T-cell epitope prediction for the extra C-terminal region of the CHIKV E2-FL protein for understanding immunogenicity differences observed in E2-FL with respect to E2- Δ C and E2- Δ NC proteins. The binding strength of T-cell epitopes to the major histocompatibility complex (MHC or HLA) molecules is a key determinant in T-cell epitope immunogenicity. Using the IEDB T-cell epitope prediction server, we identified two potential T-cell epitopes (ATVPFLLSL: 0.87 and YELTPGATV: 0.85) in the CHIKV E2-FL extra C-terminal region binding exclusively to MHC class I alleles (score value > 0.75) spanning 406–414 and 400–408 aa, respectively (Table 6). These two potential T-cell epitopes were absent in the truncated forms of E2 fragments. A study by Sreekumar et al. also identified the FLLSLICCI epitope (410–418 aa) present in the C-terminal region of the CHIKV E2-FL protein among the six T-cell epitopes identified using the EpiJen online server and implicated the importance of C416G mutation present in the cytoplasmic domain of the E2 protein.⁶¹ Including CTL epitopes in multiepitope peptide-based CHIKV vaccine is extremely beneficial as they are necessary for the clearance of intracellular pathogens by arbitrating cell-mediated immunity. It has been demonstrated that epitope-based vaccines containing epitopes recognizing multiple HLA molecules can provide the broadest possible coverage of the human population. In a separate study, the CHIKV E2 peptide KTDDSHD (spanning 57–63 aa) was predicted to be the most probable T-cell epitope and the peptide FVRTSAPCT (84–92 aa) was predicted to be the common T- and B-cell epitope having a high antigenicity.⁶² While both of these peptides were located in Domain A wherein the major secondary structure was β -strands, it was reported that Domain C of the E2 protein, which consisted of a long α -helix (362–423 aa), comprised the highly conserved “TPY” domain (398–400 aa) and the transmembrane domain (365–385 aa).⁶² In addition to the above-mentioned reports, MHC class I peptides “TAECKDKNL” and “VTWGNNEPY” were predicted to be extremely antigenic in studies by Qamar et al. and Narula et al., respectively.^{63,64} Using an *in silico* immunoinformatic approach, Khan et al. identified key mutations in the CHIKV E2 region of circulating Pakistani isolates along with the CHIKV-S27 strain and predicted a total of 18 potential CTL epitopes using bioinformatics tools.⁶⁵ Among the 18 potential CTL epitopes predicted, four epitopes were located in the extra C-terminal region of the CHIKV E2-FL protein sequence as per our study.

On analyzing the MHC class II binding of helper T-cell (HTL) epitopes present in the extra region of the CHIKV E2-FL protein, we identified a total of 37 unique T-cell epitopes, which showed binding to MHC class II alleles (Table 6). On performing immunogenicity predictions using the IEDB server for evaluating the immunogenicity of these HTL epitopes, we found two highly immunogenic peptides (MTVVVVSVAT-FILLS: 366–380 aa position; immunogenicity score: 97.67 and FILLSMVGMAAGMCM: 376–390 aa position; immunogenicity score: 90.43) in the extra C-terminal region of CHIKV E2-FL (Table 7). The peptide sequence (MTVVVVSVATFILLS; score 97.67) having the highest predicted immunogenicity was exclusively located in the extra C-terminal region of the CHIKV E2-FL protein and was absent in the two truncated E2 protein fragments. From the allele cumulative frequency distribution graphs, we found that the predicted T-cell epitope MTVVVVSVATFILLS (score 97.67) binds to 41% of MHC class II alleles, while FILLSMVGMAAGMCM (score 90.43) showed 19% binding to MHC class II alleles (Figures 12A,B). The MHC class II alleles that exhibit good binding affinities ($IC_{50} < 500$ nM) with these two peptides are listed in Table 8. Our results indicate that 16 MHC class II alleles can interact with MTVVVVSVATFILLS and 10 MHC class II alleles can interact with FILLSMVGMAAGMCM epitopes, present in the E2-FL protein. Since the epitopes that interact with numerous alleles are considered more efficient for imparting broad population coverage, including these two immunogenic epitopes located in the extra C-terminal region of the CHIKV E2-FL protein in a multiepitope-based anti-CHIKV vaccine may prove beneficial. Using an immune-informatics approach, a previous study identified the IMLLYPDHPTLL-SYR epitope spanning 284–298 aa in the CHIKV E2 glycoprotein as a potent HTL epitope, which could interact with five MHC class II alleles ($IC_{50} < 250$).⁶⁶ Interestingly, a proteome-wide screening study by Teo et al. identified a dominant CD4⁺ T-cell epitope in the N-terminal E2EP3 region (spanning 1–19 aa) of the E2 protein, by performing ELISPOT assays on splenocytes obtained from CHIKV-infected mice.⁶⁷ It is likely that a vaccine containing such a type of peptide epitope will have a large population coverage and will provide future direction for vaccine design and development against chikungunya infection. Furthermore, a multiepitope-based peptide can be used as an alternate antigen to CHIKV E2-FL for immunodiagnosis of CHIKV infections with high sensitivity and specificity.⁶⁸ In summary, we identified the potential B- and T-cell epitopes present in the extra C-terminal region of the CHIKV E2-FL protein. Since the extra C-terminal region of the CHIKV E2-FL protein contains extra immunogenic epitopes that are absent in the truncated fragments and can bind with a more diverse human MHC allele repertoire, it is expected to significantly enhance the effective immune response and therefore may be advantageous to be included when designing multiepitope vaccines or immunotherapies for CHIKV.

4. CONCLUSIONS

Several reports have shown that the CHIKV E2 protein can serve as an effective subunit vaccine candidate against chikungunya infection. This study provides a detailed methodology optimized for cloning, expression, and purification of CHIKV E2-FL and E2 truncated proteins using a prokaryotic expression system. Using an *E. coli*-based expression system is a

cost-effective option and makes the process amenable to inexpensive scale-up. The CHIKV E2 proteins were purified using Ni-NTA chromatography and were refolded *in vitro* for obtaining proteins in native conformation (devoid of any aggregates or protein precipitates). Structural analysis performed using CD and FTIR spectroscopies confirmed the presence of CHIKV E2 proteins in their near-native state. The biological function of the refolded and purified recombinant CHIKV E2-FL protein was confirmed by its ability to generate high titers of E2-specific antibodies in BALB/c mice. The recombinant CHIKV E2 proteins expressed in *E. coli* retained their immunogenicity and could induce high antibody titers in BALB/c mice, as evaluated using E2-specific ELISA. By performing B-cell and T-cell epitope analyses using various *in silico* approaches, our study identified highly immunogenic unique peptides present in the CHIKV E2-FL protein. Specifically, both the N-terminal region (1–32 aa) and the C-terminal region (366–423 aa) present in the CHIKV E2-FL protein demonstrated the presence of unique highly immunogenic peptides. We anticipate that our predicted B-cell and T-cell epitopes could aid the design of a prospective anti-CHIKV multiepitope vaccine. In conclusion, the results outlined in this study could be valuable for the development of novel and effective multiepitope subunit vaccine strategies for prevention of CHIKV infections and also help in producing purified antigens in large amounts for use in anti-CHIKV diagnostic and/or serological tests. Furthermore, this approach of expressing and purifying the CHIKV E2 protein in *E. coli* with a high yield may also offer a promising method for the production of other viral recombinant proteins.

5. EXPERIMENTAL SECTION

5.1. Materials. Chikungunya virus strain isolate (Ind-06-Guj, Gen Bank Accession No. JF274082) was kindly provided by Dr. Sudhanshu Vрати (RCB, Faridabad, India). The plasmid vector pQE-30 Xa along with the bacterial host *E. coli* strains XL1-Blue and SG13009 were kindly provided by Dr. Devinder Sehgal (NII, New Delhi, India). Media for bacterial growth was purchased from Himedia Laboratories, India. All restriction enzymes, PCR components, and DNA Ligase were procured from NEB (New England Biolabs). Oligos were synthesized from Eurofins, India. Ni-NTA resin was purchased from Qiagen, Germany. Dialysis tubing was obtained from Spectrum Labs. Antibodies for Western blotting were purchased from ThermoFisher Scientific. All chemicals were procured from Sigma.

5.2. PCR Amplification and Cloning of E2 Gene Constructs. The nucleotide sequence of CHIKV E2 was downloaded from the NCBI database (Accession No. JF274082) and used as a template to design gene-specific primers. Total RNA was isolated from the chikungunya viral lysate (Ind-06-Guj) using the High Pure Viral RNA kit as per the manufacturer's instructions (Roche Life Science). Total RNA was used for cDNA synthesis following the commercial protocol and the Oligo-dT primer (Verso cDNA Synthesis kit, Thermo Fisher). The CHIKV E2-FL nucleotide sequence (1269 bp; corresponding to 1–423 amino acid residues) was PCR-amplified using high-fidelity Phusion DNA Polymerase (NEB, England) and gene-specific forward (5'-CCCCGGATCCAGCACCAAGGACAACCTTCAATG) and reverse (5'-CCCCCAAGCTTCGCTTTAGCTGTTCTGATGCAG) primers, respectively (Table 1). The underlined nucleotides in forward and reverse primer sequences signify

*Bam*HI and *Hind*III restriction enzyme sites, respectively. The 25 μ L PCR reaction mix comprised 1 \times DNA polymerase buffer, 200 μ M dNTP mix, 0.2 μ M of each primer, 5 μ L of cDNA, and 1 U of Phusion DNA polymerase. PCR was performed using a programmable thermal cycler (GeneAmp PCR system 2720; Applied Biosystems) starting with an initial denaturation step at 95 $^{\circ}$ C for 2 min. This was followed by 25 cycles consisting of denaturation at 95 $^{\circ}$ C for 1 min, annealing at 60 $^{\circ}$ C for 1 min, and an extension at 72 $^{\circ}$ C for 1 min. A final extension step was conducted at 72 $^{\circ}$ C for 10 min. The PCR amplicon (1269 bp) was subsequently digested with *Bam*H1 and *Hind*III restriction enzymes. The digested product was ligated into *Bam*H1-*Hind*III digested *E. coli* expression vector pQE-30 Xa using T4 DNA ligase. The ligated product was transformed in the *E. coli* XL1-Blue strain. Positive clones were identified by restriction analysis and confirmed by sequencing.

The truncated CHIKV E2- Δ C fragment (1095 bp; corresponding to 1–365 amino acids) was obtained through PCR amplification using the full-length E2 plasmid DNA as the template along with gene-specific forward (5'-CCCCGGATCCGAACGCATCAGAAATGAAGCGAC) and reverse (5'-CCCCAAGCTTAGTAGGGTACAGCTCA-TAATAATAC) primers. The truncated CHIKV E2- Δ NC fragment (993 bp; corresponding to 35–365 amino acids) was obtained through PCR amplification using CHIKV E2-FL plasmid DNA as the template along with gene-specific forward (5'-CCCCGGATCCGAACGCATCAGAAATGAAGCGAC) and reverse (5'-CCCCAAGCTTAGTAGGGTACAGCT-CATAATAATAC) primers, respectively. The PCR components and cycling conditions used were the same as mentioned above. The amplicons obtained were digested with *Bam*H1 and *Hind*III enzymes and cloned into the digested pQE-30 Xa vector. Positive clones were identified by restriction analysis and confirmed by sequencing.

5.3. Expression of Recombinant E2 Proteins. For expression purposes, the CHIKV E2-FL and truncated E2 constructs were transformed in the *E. coli* expression strain SG13009 (Qiagen). Bacterial cultures were grown in a Luria-Bertani medium containing ampicillin (100 μ g/mL) and kanamycin (25 μ g/mL) at 37 $^{\circ}$ C overnight with shaking at 220 rpm. The secondary cultures were induced at 0.2 OD₆₀₀ by adding 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) for 18 h at 18 $^{\circ}$ C. Bacteria were harvested by centrifugation at 8000 rpm for 30 min at 4 $^{\circ}$ C. Uninduced and induced bacterial pellets were analyzed on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized using Coomassie brilliant blue R-250 staining. For determining protein solubility, bacterial cell pellets obtained after induction were lysed using a Lysis buffer (containing 25 mM pH 7.3, 300 mM NaCl, and 5 mM β -mercaptoethanol) supplemented with 1 \times Protease inhibitor cocktail (Roche). The lysate was sonicated briefly and centrifuged at 12 000 rpm for 30 min at 4 $^{\circ}$ C. The soluble (supernatant) and insoluble (pellet) protein fractions obtained were analyzed on 10% SDS-PAGE gel. The expressed protein found in the insoluble fraction was processed for subsequent purification steps.

5.4. Purification of Recombinant E2 Proteins. The recombinant CHIKV E2-FL and truncated E2 proteins were purified using Ni-NTA affinity chromatography under denaturing conditions with some modifications from the manufacturer's protocol (Qiagen, Germany). Briefly, the cell pellet obtained after induction was lysed in a Lysis buffer (containing 8 M urea, 25 mM Tris pH 7.3, 300 mM NaCl, and

5 mM β -mercaptoethanol) supplemented with 1 \times Protease inhibitor cocktail (Roche). Cell lysates were prepared by 20 sonication cycles of a 10 s duration pulse with a 10 s time interval between pulses. The lysate was centrifuged at 12 000 rpm for 30 min at 4 $^{\circ}$ C, and the supernatant containing the fusion protein was loaded onto a column containing 1 mL of equilibrated Ni-NTA resin and allowed to bind at room temperature for 15 minutes. The column was washed with wash buffers (lysis buffer with 8 M urea supplemented with increasing concentrations of imidazole (20, 50, and 100 mM)). Finally, the column was washed with an elution buffer (lysis buffer with 8 M urea, supplemented with 250 mM imidazole). The washed and eluted fractions were analyzed on 10% SDS-PAGE for purity estimation. During column washing steps in Ni-NTA chromatography, wash buffers had 0.1% Triton X-114 added as the default as per the manufacturer's instructions, for removing endotoxin contamination. The endotoxin levels in all immunogens were found to be between 0.5 and 0.1 EU/mL, using the Pierce LAL Chromogenic Endotoxin Quantitation kit (Thermo scientific).

5.5. Protein Refolding. The purified recombinant CHIKV E2-FL and E2 truncated protein fractions were refolded using a single step or the stepwise dialysis method. In the single-step dialysis procedure carried out for 6 h at 21 $^{\circ}$ C with continuous stirring, a visible protein precipitate was observed. Subsequently, refolding was optimized using a stepwise dialysis procedure. Briefly, eluted fractions were pooled and dialyzed against refolding buffer (containing 20 mM Tris pH 7.3, 5 mM DTT, 400 mM NaCl, and 5 mM EDTA), which had decreasing amounts of urea in subsequent buffer changes. The concentration of urea was decreased gradually (6, 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.015, and 0 M), keeping the concentration of the rest of the constituents constant. Each dialysis step was carried out at room temperature (21 $^{\circ}$ C) at pH 7.3 for 3 h with gentle stirring. All dialysis procedures were performed at 4 $^{\circ}$ C once the urea concentration was decreased to 2 M. While performing urea-free dialysis in the last step, the dialysis buffer was supplemented with 20% glycerol. The refolded protein samples were removed from the dialysis bags and centrifuged to remove any residual aggregates. The dialyzed and undialyzed protein samples were stored at -20 $^{\circ}$ C and analyzed on 10% SDS-PAGE gel. The concentration of the refolded protein was estimated spectrophotometrically by the BCA protein microassay (Genetix) using bovine serum albumin (BSA) as the standard. The *in vitro* refolding yield was calculated as per the following formula

$$\text{refolding yield} = \frac{\text{total recovered refolded protein (mg)}}{\text{initial protein used for refolding (mg)}} \times 100$$

5.6. Western Blotting. Purified N-terminal histidine-tagged full-length and truncated E2 fusion proteins were separated on a 10% SDS-PAGE gel along with protein marker, positive control (histidine-tagged fusion protein), and negative control (bovine serum albumin, BSA) proteins and transferred to a nitrocellulose membrane (Bio-Rad Laboratories). The membrane was blocked using 3% (w/v) BSA in Tris-buffered saline (TBS) for 1 h at room temperature. Subsequently, the membrane was washed four times with 1 \times TBS containing 0.05% Tween-20 (TBST). Next, the membrane was incubated with the anti-His monoclonal antibody (Santa Cruz Biotechnology) at 1:1000 dilution for 1 h at room temperature.

After washing four times with TBST, the membrane was incubated for 1 h at room temperature with HRP-conjugated goat antimouse IgG (Santa Cruz Biotechnology) at 1:5000 dilution. After washing four times with TBST and once with TBS, the membrane was incubated with a 3,3'-diaminobenzidine (DAB) substrate solution (0.5 mg/mL) at room temperature. Color development was stopped by washing with water, following which the blots were air-dried and stored.

5.7. Circular Dichroism. To evaluate the secondary structure content of purified recombinant CHIKV E2 proteins, both the unfolded and refolded CHIKV E2-FL, E2- Δ C, and E2- Δ NC proteins were subjected to CD analysis using a Chirascan Circular Dichroism Spectrometer (Applied Photophysics Ltd., Surrey KT22 7PB, United Kingdom). CD spectra were collected using a 1 mm quartz cell under constant nitrogen purge between 190 and 250 nm in 1 nm wavelength steps and an average time of 2.0 s at 18 °C. Protein samples at concentrations of 0.5 mg/mL were analyzed in a 20 mM Tris, pH 7.3 buffer containing 400 mM NaCl, 5 mM EDTA, and 20% glycerol. For each sample, five scans were collected and averaged. The final average spectrum of all of the samples was estimated by subtracting the baseline spectrum of the corresponding buffer. The data was analyzed by the CAPITO web tool (<https://capito.uni-jena.de>). Thereafter, the secondary structure-specific graphs were obtained by plotting the molar ellipticity against wavelength. Recorded CD data in millidegrees were converted to molar ellipticity as per the following formula: molar ellipticity (θ) = ($m^\circ \times M$)/(10 \times L \times C), where θ (theta) is the molar ellipticity in deg cm²/dmol, m° is the recorded ellipticity in millidegrees, M is the mean residual weight (g/mol), L is the path length in cm, and C is the concentration in g/L.⁶⁹ Smoothing of the raw CD data was performed using the Savitzky–Golay filter on CAPITO software online.

5.8. FTIR Spectroscopy. The secondary structure elements of the denatured and refolded E2 protein samples were further analyzed by Fourier transform infrared (FTIR) spectroscopy. A PerkinElmer Spectrum Two FTIR spectrometer was used to measure the spectrum in the spectral range of 400–4000 cm⁻¹ at a resolution of 8 cm⁻¹ and an average of 16 scans, using potassium bromide (KBr) pellets. FTIR spectroscopy was performed at the Department of Chemistry, IIT Roorkee.

5.9. Mice Immunization. Five- to six-week-old inbred male BALB/c mice were obtained from CDRI (Lucknow, India) and were housed in the Institute Animal Facility at IIT Roorkee (Uttarakhand, India). All animals were maintained at a 12:12 h light/dark cycle at 25 °C and were provided with a pellet diet and water *ad libitum*. Approval from the Institutional Animal Ethics Committee was obtained for all experimental procedures involving mice for this study. Different groups of BALB/c mice ($n = 5$) were immunized subcutaneously with 10 μ g of purified recombinant CHIKV E2-FL and E2 truncated proteins along with 50 μ g of alum (Pierce) as the adjuvant in PBS. After 3 weeks (day 21) and 6 weeks (day 42), mice were boosted with the same dose of antigen and adjuvant. Sham-immunized mice received an equal amount of adjuvant in saline on day 0, day 21, and day 42. Mice ($n = 5$) were bled retro-orbitally every week, at indicated timepoints (days 7, 14, 19, 28, 35, 42, 49, 56, 63). The isolated blood samples were allowed to clot for 2–3 h at room temperature and later centrifuged to separate serum. The serum samples were

removed by aspiration and stored at -20 °C for further analysis.

5.10. E2-Specific ELISA. For determining E2-specific antibody titers, 96-well ELISA plates (Nunc MaxiSorp, Thermo) were coated with 5 μ g/mL recombinant full-length E2 protein in a carbonate-bicarbonate buffer overnight at 4 °C. All blocking steps were performed using 5% milk protein in PBS for 1 h at 37 °C, and plates were washed with 0.05% Tween-20 in PBS (PBST) before use. The plates were then incubated with serially diluted serum samples obtained from mice immunized with CHIKV E2-FL, E2- Δ C, and E2- Δ NC proteins along with commercially available monoclonal antibody (18H01) purchased from The Native Antigen (Oxford, United Kingdom) as a positive control. Serum samples were incubated for 1 h at 37 °C, following which the plates were washed, and CHIKV E2-specific antibody levels were detected with the HRP-conjugated goat antimouse IgG secondary antibody (Southern Biotechnology) (1:5000 dilution) for 1 h at 37 °C. Plates were subsequently developed with 0.4 mg/mL o-phenylenediamine dihydrochloride (SRL, India) dissolved in citrate-phosphate buffer (pH 5.0). The reaction was stopped with 2 N H₂SO₄, and optical density at 490 nm was measured using a microplate reader (Tecan, USA). Titers were defined as the highest dilution of serum that gave an optical density at least twice the mean background reading (wells with all reagents except sera).

5.11. Epitope Analysis. **5.11.1. B-Cell Epitope Analysis.** The B-cell epitopes of CHIKV E2-FL, E2- Δ C, and E2- Δ NC proteins are delineated as per previously published reports, which included experimental validation of these epitopes.^{24,70} Since B-cell epitopes are characterized by four parameters, namely, surface accessibility, antigenicity, flexibility, and hydrophilicity, tertiary 3D structure modeling was performed using both SWISS-MODEL (<https://swissmodel.expasy.org>) and I-TASSER (<http://zhanglab.cmb.med.umich.edu/I-TASSER/download/>) for CHIKV E2-FL, E2- Δ C, and E2- Δ NC antigens based on available structure templates. The modeled 3D structures of the three CHIKV E2 proteins were superimposed using Pymol 3D structure visualization software (<http://www.pymol.org/>) for comparison of their respective epitope structures and surface accessibility. The physical and chemical properties {including amino acid composition, molecular weight, theoretical isoelectric point, instability coefficient, and grand average of hydropathicity (GRAVY)} of the three different CHIKV E2 proteins and their respective epitopes were analyzed using ProtParam (<http://web.expasy.org/protparam/>). Additionally, two freely available online epitope prediction servers, namely, ABCpred (https://webs.iiitd.edu.in/raghava/abcpred/ABC_submission.html) and Bcepred (<http://www.imtech.res.in/raghava/bcepred/>), were used to predict the B-cell epitopes using their default threshold values for CHIKV E2-FL, E2- Δ C, and E2- Δ NC antigens used in this study.

5.11.2. T-Cell Epitope Prediction. The MHC I binding affinity of T-cell epitopes was predicted using the default IEDB-recommended 2020.09 NetMHCpan EL 4.1 method (<http://tools.iedb.org/mhci/>), and the MHC II binding affinity of T-cell epitopes was identified using the default IEDB-recommended 2.22 method (<http://tools.iedb.org/mhcii/>). Further, the immunogenicity values of the *in silico*-predicted MHC class II binding peptides of the E2-FL and truncated CHIKV E2 proteins used in the study were calculated using the IEDB CD4 T-cell immunogenicity

(<http://tools.iedb.org/CD4episcore/>) tool against seven reference sets of human HLA alleles.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.1c05975>.

FTIR spectra results for unfolded CHIKV E2-FL, E2-ΔC, and E2-ΔNC proteins (Figure S1) (PDF)

(PDF)

■ AUTHOR INFORMATION

Corresponding Author

Soma Rohatgi – Department of Biosciences and Bioengineering, Indian Institute of Technology Roorkee, Roorkee 247667 Uttarakhand, India; orcid.org/0000-0002-0953-3132; Email: soma.rohatgi@bt.iit.ac.in

Authors

Manisha Shukla – Department of Biosciences and Bioengineering, Indian Institute of Technology Roorkee, Roorkee 247667 Uttarakhand, India

Pankaj Chandley – Department of Biosciences and Bioengineering, Indian Institute of Technology Roorkee, Roorkee 247667 Uttarakhand, India

Suman Tapryal – Department of Biotechnology, Central University of Rajasthan, Ajmer 305817 Rajasthan, India; orcid.org/0000-0002-7956-8509

Narendra Kumar – Jaypee University of Information Technology, Solan 173234, India

Sulakshana P. Mukherjee – Department of Biosciences and Bioengineering, Indian Institute of Technology Roorkee, Roorkee 247667 Uttarakhand, India; orcid.org/0000-0002-9420-6215

Complete contact information is available at: <https://pubs.acs.org/10.1021/acsomega.1c05975>

Author Contributions

M.S.: methodology, investigation, and original draft preparation. P.C.: writing, reference collection, and proofreading. S.T.: methodology. N.K.: methodology. S.P.M.: methodology. S.R.: conceptualization, methodology, supervision, writing, and reviewing. All authors contributed to the manuscript. All authors have read and agreed to the final version of the manuscript.

Funding

This work was supported by the Ramalingaswami grant (Dept. of Biotechnology, Govt. of India) and the Early Career Research Award (Science and Engineering Research Board, Govt. of India) to S.R. The authors acknowledge the DBT-JRF Fellowship grant to MS, ICMR-JRF Fellowship grant to P.C, and research and infrastructural facilities at IIT-Roorkee.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank Ravikant Ranjan for technical assistance and suggestions on the manuscript.

■ REFERENCES

- (1) Morrison, T. E. Reemergence of Chikungunya Virus. *J. Virol.* **2014**, *88*, 11644–11647.
- (2) Schwameis, M.; Buchtele, N.; Wadowski, P. P.; Schoergenhofer, C.; Jilma, B. Chikungunya vaccines in development. *Hum. Vaccin. Immunother.* **2016**, *12*, 716–731.
- (3) Powers, A. M.; Logue, C. H. Changing patterns of chikungunya virus: re-emergence of a zoonotic arbovirus. *J. Gen. Virol.* **2007**, *2363*–2377.
- (4) Chen, C. I.; Clark, D. C.; Pesavento, P.; Lerche, N. W.; Luciw, P. A.; Reisen, W. K.; Brault, A. C. Comparative pathogenesis of epidemic and enzootic Chikungunya viruses in a pregnant Rhesus macaque model. *Am. J. Trop. Med. Hyg.* **2010**, *83*, 1249–1258.
- (5) Thiboutot, M. M.; Kannan, S.; Kawalekar, O. U.; Shedlock, D. J.; Khan, A. S.; Sarangan, G.; Srikanth, P.; Weiner, D. B.; Muthumani, K. Chikungunya: A potentially emerging epidemic? *PLoS Neglected Trop. Dis.* **2010**, *4*, No. e623.
- (6) Badawi, A.; Gwan, S.; Vasileva, D.; Yaghoubi, S. International Journal of Infectious Diseases Prevalence of chronic comorbidities in chikungunya: A systematic review and meta-analysis. *Int. J. Infect. Dis.* **2018**, *67*, 107–113.
- (7) Borgherini, G.; Poubeau, P.; Jossaume, A.; Gouix, A.; Cotte, L.; Michault, A.; Arvin-Berod, C.; Paganin, F. Persistent arthralgia associated with chikungunya virus: A study of 88 adult patients on Reunion Island. *Clin. Infect. Dis.* **2008**, *47*, 469–475.
- (8) Phommanivong, V.; Kanda, S.; Shimono, T.; Lamaningao, P.; Darcy, A. W.; Mishima, N.; Phaytanavanh, B.; Nishiyama, T. Co-circulation of the dengue with chikungunya virus during the 2013 outbreak in the southern part of Lao PDR. *Trop. Med. Health* **2016**, *44*, 1–10.
- (9) Simon, F.; Javelle, E.; Oliver, M.; Leparac-Goffart, I.; Marimoutou, C. Chikungunya virus infection. *Curr. Infect. Dis. Rep.* **2011**, *13*, 218–228.
- (10) Schilte, C.; Staikovsky, F.; Couderc, T.; Madec, Y.; Carpentier, F.; Kassab, S.; Albert, M. L.; Lecuit, M.; Michault, A. Chikungunya Virus-associated Long-term Arthralgia: A 36-month Prospective Longitudinal Study. *PLoS Neglected Trop. Dis.* **2013**, *7* DOI: [10.1371/journal.pntd.0002137](https://doi.org/10.1371/journal.pntd.0002137).
- (11) Gérardin, P.; Barau, G.; Michault, A.; Bintner, M.; Randrianaivo, H.; Choker, G.; Lenglet, Y.; Touret, Y.; Bouveret, A.; Grivard, P.; et al. Multidisciplinary prospective study of mother-to-child chikungunya virus infections on the island of La Réunion. *PLoS Med.* **2008**, *5*, No. e60.
- (12) Renault, P.; Solet, J. L.; Sissoko, D.; Balleydier, E.; Larrieu, S.; Filleul, L.; Lassalle, C.; Thiria, J.; Rachou, E.; De Valk, H.; et al. A major epidemic of chikungunya virus infection on Reunion Island, France, 2005–2006. *Am. J. Trop. Med. Hyg.* **2007**, *77*, 727–731.
- (13) Schwartz, O.; Albert, M. L. Biology and pathogenesis of chikungunya virus. *Nat. Rev. Microbiol.* **2010**, *8*, 491–500.
- (14) Robinson, M. C. An epidemic of virus disease in Southern Province, Tanganyika Territory, in 1952–53 I. Clinical Features. *Trans. R. Soc. Trop. Med. Hyg.* **1955**, *49*, 28–32.
- (15) Petersen, L. R.; Powers, A. M. Chikungunya: Epidemiology. *F1000Research* **2016**, *5*, 1–8.
- (16) Thiberville, S. D.; Moyen, N.; Dupuis-Maguiraga, L.; Nougairede, A.; Gould, E. A.; Roques, P.; de Lamballerie, X. Chikungunya fever: Epidemiology, clinical syndrome, pathogenesis and therapy. *Antiviral Res.* **2013**, *99*, 345–370.
- (17) Zeller, H.; Van Bortel, W.; Sudre, B. Chikungunya: Its history in Africa and Asia and its spread to new regions in 2013–2014. *J. Infect. Dis.* **2016**, *214*, S436–S440.
- (18) Khan, A. H.; Morita, K.; Parquet, C.; Hasebe, F.; Mathenge, E. G. M.; Igarashi, A. Complete nucleotide sequence of chikungunya virus and evidence for an internal polyadenylation site. *J. Gen. Virol.* **2002**, *3075*–3084.
- (19) Voss, J. E.; Vaney, M. C.; Duquerroy, S.; Vonrhein, C.; Girard-Blanc, C.; Crublet, E.; Thompson, A.; Bricogne, G.; Rey, F. A. Glycoprotein organization of Chikungunya virus particles revealed by X-ray crystallography. *Nature* **2010**, *468*, 709–712.

- (20) Weger-Lucarelli, J.; Aliota, M. T.; Kamlangdee, A.; Osorio, J. E. Identifying the Role of E2 Domains on Alphavirus Neutralization and Protective Immune Responses. *PLoS Neglected Trop. Dis.* **2015**, *9*, No. e0004163.
- (21) Akahata, W.; Nabel, G. J. A Specific Domain of the Chikungunya Virus E2 Protein Regulates Particle Formation in Human Cells: Implications for Alphavirus Vaccine Design. *J. Virol.* **2012**, *86*, 8879–8883.
- (22) Kam, Y.-W.; Lee, W. W. L.; Simarmata, D.; Harjanto, S.; Teng, T.-S.; Tolou, H.; Chow, A.; Lin, R. T. P.; Leo, Y.-S.; Renia, L.; et al. Longitudinal Analysis of the Human Antibody Response to Chikungunya Virus Infection: Implications for Serodiagnosis and Vaccine Development. *J. Virol.* **2012**, *86*, 13005–13015.
- (23) Goh, L. Y. H.; Hobson-Peters, J.; Prow, N. A.; Gardner, J.; Bielefeldt-Ohmann, H.; Pyke, A. T.; Suhrbier, A.; Hall, R. A. Neutralizing monoclonal antibodies to the E2 protein of chikungunya virus protects against disease in a mouse model. *Clin. Immunol.* **2013**, *149*, 487–497.
- (24) Chua, C. L.; Chan, Y. F.; Sam, I. C. Characterisation of mouse monoclonal antibodies targeting linear epitopes on Chikungunya virus E2 glycoprotein. *J. Virol. Methods* **2014**, *195*, 126–133.
- (25) Lum, F.-M.; Teo, T.-H.; Lee, W. W. L.; Kam, Y.-W.; Rénia, L.; Ng, L. F. P. An Essential Role of Antibodies in the Control of Chikungunya Virus Infection. *J. Immunol.* **2013**, *190*, 6295–6302.
- (26) Clayton, A. M. Monoclonal antibodies as prophylactic and therapeutic agents against chikungunya virus. *J. Infect. Dis.* **2016**, *214*, S506–S509.
- (27) Goyal, M.; Chauhan, A.; Goyal, V.; Jaiswal, N.; Singh, S.; Singh, M. Recent development in the strategies projected for chikungunya vaccine in humans. *Drug Des., Dev. Ther.* **2018**, *12*, 4195–4206.
- (28) Gao, S.; Song, S.; Zhang, L. Recent Progress in Vaccine Development Against Chikungunya Virus. *Front. Microbiol.* **2019**, *10*, No. 2881.
- (29) Verma, A.; Chandele, A.; Nayak, K.; Kaja, M. K.; Arulandu, A.; Lodha, R.; Ray, P. High yield expression and purification of Chikungunya virus E2 recombinant protein and its evaluation for serodiagnosis. *J. Virol. Methods* **2016**, *235*, 73–79.
- (30) Corrêa, D.; Ramos, C. The use of circular dichroism spectroscopy to study protein folding, form and function. *Afr. J. Biochem. Res.* **2009**, *3*, 164–173.
- (31) Kong, J.; Yu, S. Fourier transform infrared spectroscopic analysis of protein secondary structures. *Acta Biochim. Biophys. Sin.* **2007**, *39*, 549–559.
- (32) Kam, Y.; Lum, F.; Teo, T.; Lee, W. W. L.; Simarmata, D.; Harjanto, S.; Chua, C.; Chan, Y.; Wee, J.; Chow, A.; et al. Early neutralizing IgG response to Chikungunya virus in infected patients targets a dominant linear epitope on the E2 glycoprotein. *EMBO Mol. Med.* **2012**, 330–343.
- (33) Kam, Y. W.; Lee, W. W. L.; Simarmata, D.; Le Grand, R.; Tolou, H.; Merits, A.; Roques, P.; Ng, L. F. P. Unique epitopes recognized by antibodies induced in chikungunya virus-infected non-human primates: Implications for the study of immunopathology and vaccine development. *PLoS One* **2014**, *9*, No. e95647.
- (34) Lum, F. M.; Ng, L. F. P. Cellular and molecular mechanisms of chikungunya pathogenesis. *Antiviral Res.* **2015**, *120*, 165–174.
- (35) Hoque, H.; Islam, R.; Ghosh, S.; Rahaman, M. M.; Jewel, N. A.; Miah, M. A. Implementation of in silico methods to predict common epitopes for vaccine development against Chikungunya and Mayaro viruses. *Heliyon* **2021**, *7*, No. e06396.
- (36) Jakhar, R.; Gakhar, S. K. An immunoinformatics study to predict epitopes in the envelope protein of SARS-CoV-2. *Can. J. Infect. Dis. Med. Microbiol.* **2020**, *2020*, No. 7079356.
- (37) Wang, P.; Sidney, J.; Dow, C.; Mothé, B.; Sette, A.; Peters, B. A systematic assessment of MHC class II peptide binding predictions and evaluation of a consensus approach. *PLoS Comput. Biol.* **2008**, *4*, No. e1000048.
- (38) Kumar, M.; Sudeep, A. B.; Arankalle, V. A. Evaluation of recombinant E2 protein-based and whole-virus inactivated candidate vaccines against chikungunya virus. *Vaccine* **2012**, *30*, 6142–6149.
- (39) Khan, M.; Dhanwani, R.; Rao, P. V. L.; Parida, M. Subunit vaccine formulations based on recombinant envelope proteins of Chikungunya virus elicit balanced Th1/Th2 response and virus-neutralizing antibodies in mice. *Virus Res.* **2012**, *167*, 236–246.
- (40) Tripathi, N. K.; Priya, R.; Shrivastava, A. Production of recombinant Chikungunya virus envelope 2 protein in *Escherichia coli*. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 2461–2471.
- (41) Dudha, N.; Rana, J.; Gabrani, R.; Gupta, A.; Chaudhary, V. K.; Gupta, S. Small scale expression, solubilization, and characterization of Chikungunya virus structural proteins. *Asian J. Pharm. Clin. Res.* **2014**, *7*, 268–271.
- (42) Tripathi, N. K.; Priya, R.; Shrivastava, A. Immunogenicity of *Escherichia coli* expressed envelope 2 protein of chikungunya virus. *Bioengineered* **2014**, *5*, 198–203.
- (43) Metz, S. W.; Geertsema, C.; Martina, B. E.; Andrade, P.; Helden, J. G.; Van Oers, M. M.; Goldbach, R. W.; Vlak, J. M.; Pijlman, G. P. Functional processing and secretion of Chikungunya virus E1 and E2 glycoproteins in insect cells. *Virol. J.* **2011**, *8*, No. 353.
- (44) Chua, C. L.; Sam, I. C.; Chan, Y. F. Expression and purification of E2 glycoprotein from insect cells (Sf9) for use in serology. *Methods Mol. Biol.* **2016**, *1426*, 51–61.
- (45) Bagno, F. F.; Godói, L. C.; Figueiredo, M. M.; Rodrigues Sérgio, S. A.; de Fátima Silva Moraes, T.; de Castro Salazar, N.; Kim, Y. C.; Reyes-Sandoval, A.; Guimarães da Fonseca, F. Chikungunya E2 protein produced in *E. coli* and HEK293-T cells—comparison of their performances in ELISA. *Viruses* **2020**, *12*, No. 939.
- (46) Singh, S. M.; Panda, A. K. Solubilization and refolding of bacterial inclusion body proteins. *J. Biosci. Bioeng.* **2005**, *99*, 303–310.
- (47) Palmer, I.; Wingfield, P. T. Preparation and extraction of insoluble (Inclusion-body) proteins from *Escherichia coli*. *Curr. Protoc. Protein Sci.* **2012**, *1*, No. 70.
- (48) Tapryal, S.; Krishnan, L.; Batra, J. K.; Kaur, K. J.; Salunke, D. M. Cloning, expression and efficient refolding of carbohydrate-peptide mimicry recognizing single chain antibody 2D10. *Protein Expr. Purif.* **2010**, *72*, 162–168.
- (49) Kumar, J. S.; Khan, M.; Gupta, G.; Bhoopati, M.; Lakshmana Rao, P. V.; Parida, M. Production, characterization, and application of monoclonal antibodies specific to recombinant (E2) structural protein in antigen-capture ELISA for clinical diagnosis of chikungunya virus. *Viral Immunol.* **2012**, *25*, 153–160.
- (50) Metz, S. W.; Martina, B. E.; van den Doel, P.; Geertsema, C.; Osterhaus, A. D.; Vlak, J. M.; Pijlman, G. P. Chikungunya virus-like particles are more immunogenic in a lethal AG129 mouse model compared to glycoprotein E1 or E2 subunits. *Vaccine* **2013**, *31*, 6092–6096.
- (51) Bréhin, A.-C.; Rubrecht, L.; Navarro-Sanchez, M. E.; Maréchal, V.; Frenkiel, M. P.; Lapalud, P.; Laune, D.; Sall, A. A.; Desprès, P. Production and characterization of mouse monoclonal antibodies reactive to Chikungunya envelope E2 glycoprotein. *Virology* **2008**, *371*, 185–195.
- (52) Fong, R. H.; Banik, S. S. R.; Mattia, K.; Barnes, T.; Tucker, D.; Liss, N.; Lu, K.; Selvarajah, S.; Srinivasan, S.; Mabila, M.; et al. Exposure of Epitope Residues on the Outer Face of the Chikungunya Virus Envelope Trimer Determines Antibody Neutralizing Efficacy. *J. Virol.* **2014**, *88*, 14364–14379.
- (53) Morey, S. H.; Kashyap, R. S.; Purohit, H. J.; Taori, G. M.; Daginawala, H. F. An approach towards peptide-based antibody detection for diagnosis of Chikungunya infection. *Biomarkers* **2010**, *15*, 546–552.
- (54) Chua, C. L.; Sam, I. C.; Merits, A.; Chan, Y. F. Antigenic Variation of East/Central/South African and Asian Chikungunya Virus Genotypes in Neutralization by Immune Sera. *PLoS Neglected Trop. Dis.* **2016**, *10*, No. e0004960.
- (55) Chua, C. L.; Sam, I. C.; Chiam, C. W.; Chan, Y. F. The neutralizing role of IgM during early Chikungunya virus infection. *PLoS One* **2017**, *12*, No. e0171989.
- (56) Weber, C.; Büchner, S. M.; Schnierle, B. S. A Small Antigenic Determinant of the Chikungunya Virus E2 Protein Is Sufficient to

Induce Neutralizing Antibodies which Are Partially Protective in Mice. *PLoS Neglected Trop. Dis.* **2015**, *9*, No. e0003684.

(57) Basu, R.; Zhai, L.; Rosso, B.; Tumban, E. Bacteriophage Q β virus-like particles displaying Chikungunya virus B-cell epitopes elicit high-titer E2 protein antibodies but fail to neutralize a Thailand strain of Chikungunya virus. *Vaccine* **2020**, *38*, 2542–2550.

(58) Khan, N.; Bhat, R.; Jain, V.; Raghavendhar B, S.; Patel, A. K.; Nayak, K.; Chandele, A.; Murali-Krishna, K.; Ray, P. Epidemiology and molecular characterization of chikungunya virus from human cases in North India, 2016. *Microbiol. Immunol.* **2021**, *65*, 290–301.

(59) Hasan, M. A.; Khan, M. A.; Datta, A.; Mazumder, M. H. H.; Hossain, M. U. A comprehensive immunoinformatics and target site study revealed the corner-stone toward Chikungunya virus treatment. *Mol. Immunol.* **2015**, *65*, 189–204.

(60) Rodrigues, R. L.; Menezes, G. D. L.; Saivish, M. V.; Da Costa, V. G.; Pereira, M.; Moreli, M. L.; Silva, R. A. Da Prediction of MAYV peptide antigens for immunodiagnostic tests by immunoinformatics and molecular dynamics simulations. *Sci. Rep.* **2019**, *9*, No. 13339.

(61) Sreekumar, E.; Issac, A.; Nair, S.; Hariharan, R.; Janki, M. B.; Arathy, D. S.; Regu, R.; Mathew, T.; Anoop, M.; Niyas, K. P.; et al. Genetic characterization of 2006–2008 isolates of Chikungunya virus from Kerala, South India, by whole genome sequence analysis. *Virus Genes* **2010**, *40*, 14–27.

(62) Sahu, A.; Das, B.; Das, M.; Patra, A.; Biswal, S.; Kar, S. K.; Hazra, R. K. Genetic characterization of E2 region of Chikungunya virus circulating in Odisha, Eastern India from 2010 to 2011. *Infect. Genet. Evol.* **2013**, *18*, 113–124.

(63) Tahir Ul Qamar, M.; Bari, A.; Adeel, M. M.; Maryam, A.; Ashfaq, U. A.; Du, X.; Muneer, I.; Ahmad, H. I.; Wang, J. Peptide vaccine against chikungunya virus: Immuno-informatics combined with molecular docking approach. *J. Transl. Med.* **2018**, *16*, No. 298.

(64) Narula, A.; Pandey, R. K.; Khatoon, N.; Mishra, A.; Prajapati, V. K. Excavating chikungunya genome to design B and T cell multi-epitope subunit vaccine using comprehensive immunoinformatics approach to control chikungunya infection. *Infect., Genet. Evol.* **2018**, *61*, 4–15.

(65) Khan, B. A.; Saifullah; Lail, A.; Khan, S. Sub-genomic analysis of chikungunya virus e2 mutations in pakistani isolates potentially modulating b-cell & t-cell immune response. *Pakistan J. Med. Sci.* **2020**, *37*, 1–6.

(66) Bappy, S. S.; Sultana, S.; Adhikari, J.; Mahmud, S.; Khan, M. A.; Kibria, K. M. K.; Rahman, M. M.; Shibly, A. Z. Extensive immunoinformatics study for the prediction of novel peptide-based epitope vaccine with docking confirmation against envelope protein of Chikungunya virus: a computational biology approach. *J. Biomol. Struct. Dyn.* **2021**, *39*, 1139–1154.

(67) Teo, T. H.; Chan, Y. H.; Lee, W. W. L.; Lum, F. M.; Amrun, S. N.; Her, Z.; Rajarethinam, R.; Merits, A.; Röttschke, O.; Rénia, L.; et al. Fingolimod treatment abrogates chikungunya virus-induced arthralgia. *Sci. Transl. Med.* **2017**, *9*, 1–12.

(68) Bhatnagar, S.; Kumar, P.; Mohan, T.; Verma, P.; Parida, M. M.; Hoti, S. L.; Rao, D. N. Evaluation of multiple antigenic peptides based on the Chikungunya E2 protein for improved serological diagnosis of infection. *Viral Immunol.* **2015**, *28*, 107–112.

(69) Kumar, S.; Singh, V. K.; Vasam, M.; Patil, P. S.; Dhaked, R. K.; Ansari, A. S.; Lohiya, N. K.; Parashar, D.; Tapryal, S. An in vitro refolding method to produce oligomers of anti-CHIKV, E2-IgM Fc fusion subunit vaccine candidates expressed in *E. coli*. *J. Immunol. Methods* **2020**, *487*, No. 112869.

(70) Verma, P.; Bhatnagar, S.; Kumar, P.; Chattree, V.; Parida, M. M.; Hoti, S. L.; Ali, S.; Rao, D. N. Analysis of antibody response (IgM, IgG, IgG3) to Chikungunya virus using a panel of peptides derived from envelope protein for serodiagnosis. *Clin. Chem. Lab. Med.* **2014**, *52*, 297–307.