

Early neutralizing IgG response to Chikungunya virus in infected patients targets a dominant linear epitope on the E2 glycoprotein

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Chikungunya virus (CHIKV) and related arboviruses have been responsible for large epidemic outbreaks with serious economic and social impact. The immune mechanisms, which control viral multiplication and dissemination, are not yet known. Here, we studied the antibody response against the CHIKV surface antigens in infected patients. With plasma samples obtained during the early convalescent phase, we showed that the naturally-acquired IgG response is dominated by IgG3 antibodies specific mostly for a single linear epitope 'E2EP3'. E2EP3 is located at the N-terminus of the E2 glycoprotein and prominently exposed on the viral envelope. E2EP3-specific antibodies are neutralizing and their removal from the plasma reduced the CHIKV-specific antibody titer by up to 80%. Screening of E2EP3 across different patient cohorts and in non-human primates demonstrated the value of this epitope as a good serology detection marker for CHIKV infection already at an early stage. Mice vaccinated by E2EP3 peptides were protected against CHIKV with reduced viremia and joint inflammation, providing a pre-clinical basis for the design of effective vaccine against arthralgia-inducing CHIKV and other alphaviruses.

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INTRODUCTION

Chikungunya virus (CHIKV) is a virulent re-emerging human pathogen and one of the leading causes of mosquito-borne arthralgia in parts of Africa, India and Southeast Asia (Higgs, 2006; Powers & Logue, 2007). In some cases, morbidity has been unexpectedly high with extensive incapacitation, including some lethal cases (Higgs, 2006; Josseran et al, 2006; Powers & Logue, 2007; Queyriaux et al, 2008; Simon et al, 2007). CHIKV was first isolated in 1953 in Tanzania from infected patients who often developed a contorted posture owing to debilitating joint pains (Kondekar & Gogtay, 2006; Lumsden, 1955; Robinson, 1955). However, the re-emergence of CHIKV since 2005 has caused millions of cases throughout countries in and around the Indian Ocean and Southeast Asia (Powers & Logue, 2007; Renault et al, 2007; Thiboutot et al, 2010), and until now sporadic outbreaks are still ongoing in several countries inflicting naïve populations (<http://www.promedmail.org>). Singapore, for instance, experienced two successive waves of Chikungunya fever (CHIKF) outbreaks in January and August 2008 (Leo et al, 2009; Ng et al, 2009; Win et al, 2010). Although there were only 718 laboratory-confirmed cases reported in 2008 and 341 cases in 2009 (<http://www.moh.gov.sg/mohcorp/publicationsreports.aspx?id=23352>, <http://www.moh.gov.sg/mohcorp/publicationsreports.aspx?id=25254>), CHIKF remains a public threat due to the low herd immunity. Therefore, it may represent a major public health problem with severe social and economic impact.

CHIKV is one of the 29 recognized species within the genus *Alphavirus* in the *Togaviridae* family (Solignat et al, 2009). The virus contains a positive-sense, single-stranded, non-segmented ribonucleic acid (RNA) genome of approximately 11.8 kilobases in length (Strauss & Strauss, 1994), with a virion diameter of approximately 70–100 nm (Her et al, 2009; Simizu et al, 1984). The genome encodes four non-structural proteins (nsP1, nsP2, nsP3 and nsP4) and precursors of structural proteins comprising of one capsid protein (C), two envelope surface glycoproteins (E1 and E2) and two additional small proteins (E3 and 6K) (Strauss & Strauss, 1994; Teng et al, 2011). Similar to other alphaviruses, the E1 and E2 glycoproteins are postulated to be involved in mediating the fusion and interaction with host receptors during CHIKV infection (Solignat et al, 2009; Voss et al, 2010).

The virus is generally maintained in a zoonotic cycle that involves sylvatic and urban CHIKV transmission cycles (Powers, 2010). Outbreaks occurring in rural countries are mostly due to sylvatic mosquitoes that are capable of infecting both primates and humans, with primates being the primary reservoir for CHIKV (Powers & Logue, 2007). In Asia, CHIKF is identified mostly as an urban disease with humans as the primary reservoir (Jain et al, 2008; Tan et al, 2011).

CHIKV causes sudden onset of fever, rashes, arthritis and other accompanying symptoms (Lumsden, 1955; Robinson, 1955). Following the acute phase of the illness, patients develop severe chronic symptoms lasting from several weeks to months, including fatigue, incapacitating joint pain and polyarthritis (Brighton et al, 1983; Simon et al, 2007). However, as in many

other arthralgia-causing arbovirus infections, the chronic phase is observed only in a fraction of the patients (Higgs, 2006; Kondekar & Gogtay, 2006; Lumsden, 1955; Powers & Logue, 2007; Robinson, 1955). A role for both innate and adaptive immunity has been proposed (Her et al, 2010; Kam et al, 2009) but the mechanisms underlying control of viral replication and dissemination, viral clearance, and acute and chronic disease severity remain poorly defined. Although anti-CHIKV IgM and IgG antibodies have been identified in patients (Panning et al, 2008; Yap et al, 2010), the kinetics of the antibody response are not well characterized.

To date, there is no licensed vaccine against CHIKV, although potential CHIKV vaccine candidates have been tested in humans and animals with varying success (Akahata et al, 2010; Edelman et al, 2000; Harrison et al, 1967, 1971; Levitt et al, 1986; Plante et al, 2011). As a result, outbreaks are controlled predominantly by preventing the exposure of people to infected mosquito vectors (Tan et al, 2011). Therefore, there is a constant need for novel approaches in rational vaccine formulation for better efficacies with lesser drawbacks.

Here, we demonstrate the target- and isotype-specificity of the antibody response against the CHIKV surface antigens by using plasma obtained during the early convalescent phase of CHIKF patients (Kam et al, 2012; Win et al, 2010). We showed for the first time that the early neutralizing IgG3 antibodies dominating the response are mostly specific for a single epitope, 'E2EP3'. It is located at the N-terminus of the E2 glycoprotein proximal to a furin E2/E3-cleavage site that is conserved in many alphaviruses (Ozden et al, 2008). Screening across different patient cohorts suggests it to be a good serology detection marker for early CHIKV-specific immune responses. E2EP3-specific antibodies were also detected in the plasma of infected non-human primates (NHP), the preferred model system for pre-clinical studies. Moreover, mice vaccinated by the E2EP3 peptide showed reduced viremia and only minor joint inflammation after virus challenge, providing a basis for the design of effective vaccines against arthralgia-inducing CHIKV and other alphaviruses.

RESULTS

E2 glycoprotein is the dominant antigen recognized by CHIKV-infected patients

Surface proteins of RNA viruses are targets of neutralizing antibodies (Han & Marasco, 2011; Hunt et al, 2010; Murphy & Whitehead, 2011). In order to identify which of the surface proteins of CHIKV are recognized, plasma samples obtained from 30 CHIKV-patients were analysed. The samples were collected during acute median 4 days post-illness onset (pio) and early convalescent phase (median 10 days pio). Reactivity of each plasma sample was assessed by western blot using purified CHIKV virions (Fig 1A) as well as by lysates of cells transiently expressing recombinant forms of the major CHIKV surface proteins (capsid, E2 and E1 glycoproteins). Identity of the expressed protein was validated with antibodies specific for the respective surface molecule revealing also an accurate

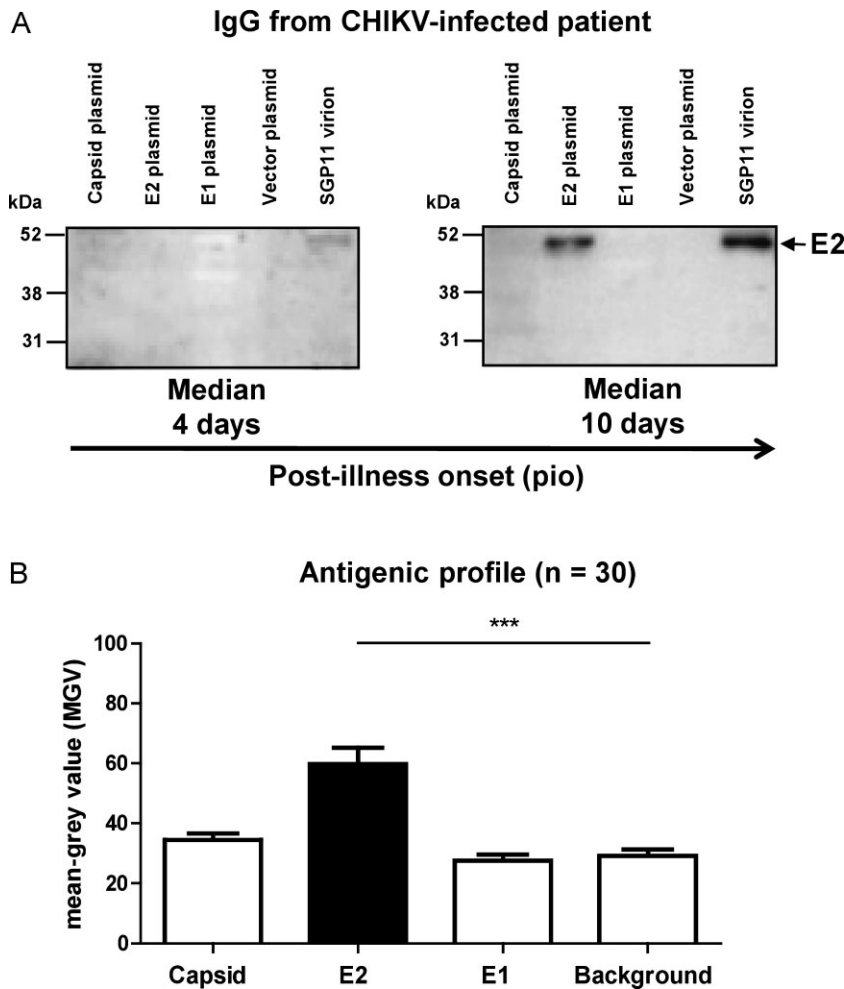


Figure 1. Antigenic recognition by CHIKV-infected patients' samples.

- A.** Total cell lysates were prepared from transiently expressed capsid protein (Capsid plasmid), E2 glycoprotein (E2 plasmid) and E1 glycoprotein (E1 plasmid). Vector transfected (Vector plasmid) cell lysates were used as negative control. Lysates and purified CHIKV virions (SGP11 virion) were subjected to SDS-PAGE gel and probed with a representative CHIKV-infected patient's plasma at a dilution of 1:2000, followed by secondary human anti-IgG-HRP. Sizes of molecular weight markers are indicated in the left part of the diagram.
- B.** Band intensities corresponding to CHIKV structural proteins (Capsid, E2 and E1) were analysed by densitometry for all patient samples ($n = 30$). Results are expressed as mean-grey value (MGV) \pm SD. Data are representative of 2 independent experiments with similar results. *** $p < 0.001$ by Kruskal–Wallis test with Dunn's post-test.

molecular weight of about 31 kDa (capsid), 52 kDa (E2) and 51 kDa (E1) (Supporting information Fig 1A).

We have previously shown that IgG can first be measured at the early convalescence time of median 10 days pio, a time point when CHIKV is no longer detectable in the blood (Kam et al, 2012). In line with this observation, no specific IgG-bands were evident when using plasma from the acute phase 4 days pio (Fig 1A, left panel), whereas a clear IgG-response was detected at median 10 day pio (Fig 1A, right panel). Notably, the plasma stained only one specific band corresponding to the E2 glycoprotein. At this time point, no major reactivity was observed for the capsid or the E1 protein, which was consistent for all 30 patients' samples. Quantification of the scanned western blots therefore revealed only for E2 bands intensities that were different from the background (Fig 1B). Thus, in line with earlier reports on other alphaviruses (Griffin, 1995; Kerr et al, 1992; Strauss et al, 1991), E2 glycoprotein is the main target in naturally-acquired immunity in infected patients who just cleared their viremia.

Epitope mapping of the E2 glycoprotein identifies a linear epitope proximal to the furin-cleavage site

In order to identify linear epitopes within the E2 glycoprotein, a peptide library consisting of overlapping peptides was scanned

with the pooled patients' plasma (Fig 2A). The library covered the entire E2 glycoprotein and consisted of 18-mer peptides, each with an overlap of 10 amino acids. Analysis of pools combining 5 consecutive peptides revealed that the IgG-response was most pronounced against the N'-terminal part of the E2 glycoprotein (Pool P1). Only some minor reactivity was detected to the other regions of the protein (Pools P2, P10 and P11) (Fig 2A). Plasma samples were next assayed with the complete set of single peptides from each of the four active pools (Fig 2B). We found that the antibodies strongly recognized the first two peptides of pool 1. In a previous study, we established that the early IgG response against CHIKV is almost exclusively driven by antibodies of the IgG3 isotype (Kam et al, 2012). A very similar picture therefore emerged when anti-IgG3 instead of anti-IgG was used for detection (Fig 2C, Supporting information Fig 1B). Although the sensitivity of the IgG3 assay is generally weaker, the two peptides of pool 1 (P1-1 and P1-2) were clearly detectable, showing a slightly stronger titer for P1-1.

The strong response against the first two peptides suggested that the epitope (termed here 'E2EP3') should be present within the overlapping part of peptides P1-1 and P1-2. The sequence alignment revealed that the overlap (STKDNFNVYKATRPYLAH) is located proximal to the furin cleavage site. The site is

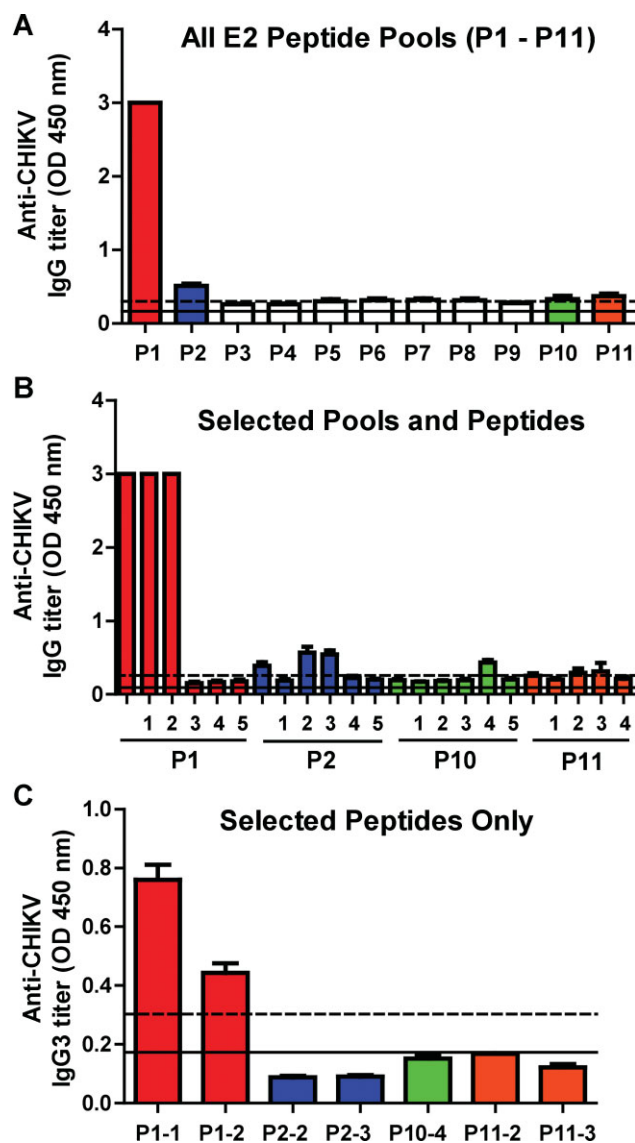


Figure 2. Epitope mapping of the E2 glycoprotein.

- A.** CHIKV-infected patient plasma pools (Median 10 days pio) were subjected to peptide-based ELISA at a dilution of 1:2000, followed by secondary human anti-IgG-HRP using pooled peptides (P1–P11).
- B.** The same set of patient plasma pools were subjected to peptide-based ELISA at a dilution of 1:2000, followed by secondary human anti-IgG-HRP using both selected peptide pools (P1, 2, 10 and 11) and individual peptides.
- C.** Selected individual peptides were re-screened with patients' plasma pools at a dilution of 1:200, followed by secondary human anti-IgG3-HRP. Black solid line represents the mean value of the healthy donors and dotted line represents the value of mean \pm 6 SD. Values above mean \pm 6 SD are considered positive. Results represent an average of two independent experiments.

required for the proteolytic generation of E2 and E3 glycoproteins from the common precursor protein (Ozden et al, 2008) and the 'furin loop' is conserved in alphaviruses (Ozden et al, 2008). The availability of the recent crystal structure of the CHIKV E1–E2 glycoprotein (Voss et al, 2010) further allowed the

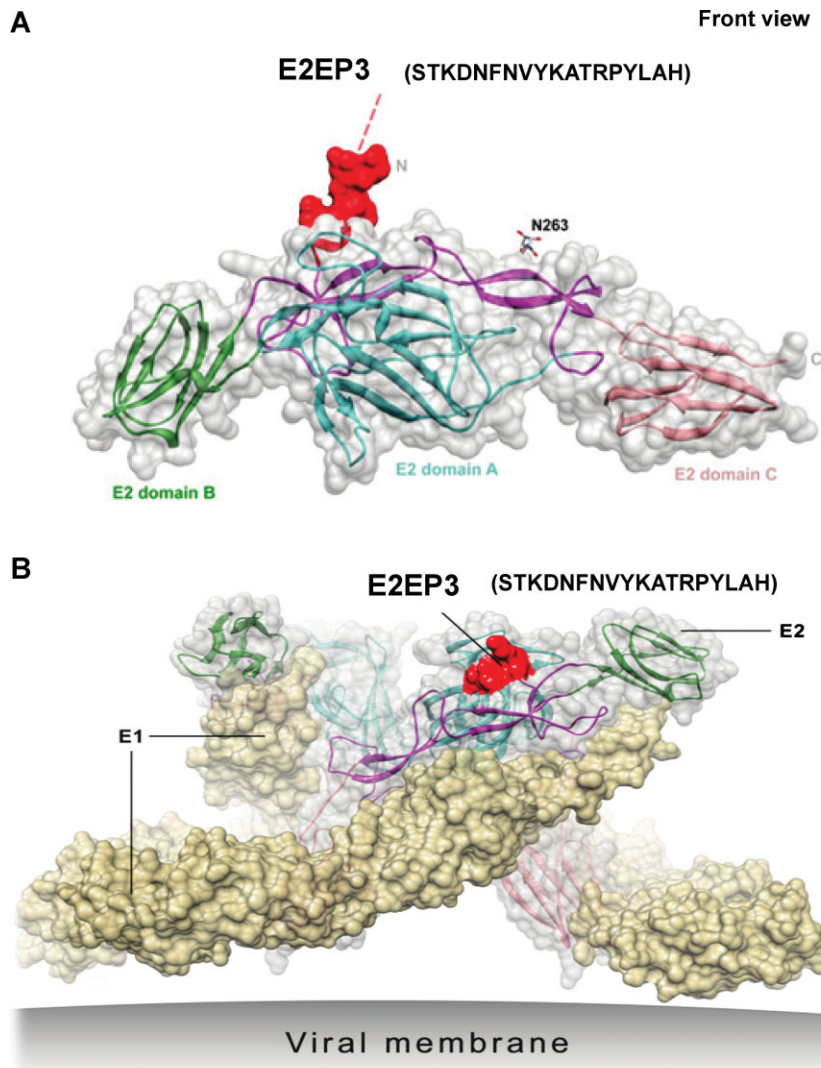
precise localization of E2EP3 epitope. In the mature E2 glycoprotein (Fig 3A), the amino acids of E2EP3 form the N-terminal part of the molecule. This region is prominently exposed on the surface of the virus, forming a stalk that points away from the virus envelope (Fig 3A and B). Control peptides from other regions of the E2 glycoprotein did not detect any reactivity from the patients' plasma (Supporting information Fig 2A). Using a library of peptides containing a series of alanine-substituted amino acids (Cunningham & Wells, 1989), we were able to identify both the core-binding region as well as the key amino acids recognized by anti-E2EP3 antibodies of patients' plasma. The result of the alanine-scan (Fig 4A and Supporting information Fig 3) was in good correlation with the crystal structure (Fig 4B). Based on this data, the core-binding region of E2EP3 comprises aa3-10 (STKDNFNVYK), which represents the exposed part of the sequence (aa1-3 were not resolved in the crystal structure).

A particularly strong abrogation of binding was observed after replacing residues K₃, N₅ and K₁₀. Their amino acid side chains are either polar (N₅) or positively charged (K₃, K₁₀), and were exposed to solvent in the crystal structure. The substitution of these amino acids reduced antibody binding to below 40% compared to the original E2EP3 peptide (Fig 4A). Detailed information on the titers against the substituted peptides is shown (Supporting information Fig 3, and a representative video of the E2EP3 epitope analysis is shown in Supporting information Video 1.

The neutralizing effect of patients' plasma is directed predominantly against E2EP3

The neutralizing capacity of CHIKV-specific antibodies in the plasma was tested *in vitro*. For this, CHIKV were pre-incubated with the pools of patients' plasma before infecting HEK 293T cells. Immunofluorescence staining followed by single-cell quantification using the Cellomics high content screen was used to assess infectivity by determining the number of CHIKV positive cells. Pooled plasma from infected patients effectively neutralized CHIKV infection. Here, the infection rate decreased to approximately 20% of total cells (Fig 5A). The addition of soluble E2EP3 peptide to the plasma, however, partially abrogated the neutralization. Blocking with E2EP3 peptide increased CHIKV infection from 20% to almost 40% (Fig 5A), verifying that antibodies to E2EP3 are strongly neutralizing.

This observation was further confirmed in experiments where E2EP3-specific IgG3 antibodies were selectively depleted. Exposure of the patients' plasma to surface-bound E2EP3 peptide completely removed all E2EP3-specific IgG3, while a partial depletion was achieved with peptides where the key amino acids K₃, N₅ and K₁₀ were alanine-substituted (E2EP3-specific IgG3 was depleted by 30% for peptide K₃A/K₁₀A, and by 15% for peptide K₃A/N₅A/K₁₀A) (Fig 5B). The impact of the complete or partial depletion of E2EP3-specific IgG3 antibodies was then tested by comparing the titers of the plasma pools on whole virus (Fig 5C). The removal of E2EP3-specific antibodies reduced the total anti-CHIKV IgG3 titer by almost 80%. The partial removal by peptide K₃A/K₁₀A decreased the titer by 40%, while peptide K₃A/N₅A/K₁₀A decreased by 20% (Fig 5C). The drastic reduction in the titer indicates that anti-E2EP3 antibodies

**Figure 3. Localization of the E2EP3 epitope.**

- A.** Schematic diagram showing the localization of the E2 glycoprotein specific epitope (E2EP3, in red colour) in the E2 glycoprotein alone based on structural data retrieved from PDB records: 3N44. Tertiary structure of E2 glycoprotein is arranged into three structural domains (E2 domain A, amino terminal; E2 domain B, centre; E2 domain C, carboxyl terminal).
- B.** Schematic diagram showing the localization of E2EP3 in the protein complex situated at the surface of the virus based on structural data retrieved from PDB records: 2XFB. Spatial arrangement of E1 glycoprotein (in pale yellow colour) and E2 glycoprotein (in light grey colour) on the viral membrane surface are indicated accordingly.

make up a substantial fraction of the total CHIKV specific IgG3 (Fig 5C).

The removal of E2EP3-specific IgG3 also directly translated into a reduced neutralization capacity of the plasma pools (Fig 5D). Depletion of plasma with E2EP3 partly restored virus infectivity from around 20% to more than 50%. As expected, only a gradual decrease of the neutralizing efficacy was observed for the alanine-substituted E2EP3 peptides K₃A/K₁₀A and K₃A/N₅A/K₁₀A (Fig 5D). Thus, during early convalescence, E2EP3 specific IgG3 antibodies largely mediate the neutralizing effect in patients' plasma.

E2EP3 specific IgG3 is a common marker of early CHIKV-infection

At median 10 days pio, almost all of the patients from this cohort were sero-positive for E2EP3 IgG3 antibodies (Supporting information Fig 4). To further validate the specificity and versatility of E2EP3 as a suitable early detection target, we screened plasma samples from another 36 CHIKV-infected patients collected from a separate cohort together with plasma

obtained from 11 healthy donors (Fig 6). Here, plasma were again collected during the early convalescent phase (median 10 days pio) and tested for anti-E2EP3 IgG3 antibodies by ELISA (Fig 6B). Whole virus was used as a reference (Fig 6A). As in the previous cohort, specific E2EP3-binding was detected in virtually all CHIKV-infected patients with a clear segregation from the sero-negative healthy control donors (Fig 6A and B). Similar results were also obtained in a cohort from Malaysia where early convalescence samples of median 14 days pio were collected at outbreaks a few months later (Sam et al, 2009). Likewise, all of the patients screened were sero-positive for E2EP3, while no reactivity against the epitope was detected in healthy donors (Fig 6C and D). Thus, E2EP3 specific IgG3 antibodies appear to be a common early marker for CHIKV-infections at the population level.

E2EP3 in pre-clinical models: marker and vaccine

Non-human primates (NHP) are the most relevant and commonly used pre-clinical models for viruses (Higgs & Ziegler, 2010; Labadie et al, 2010; Liu et al, 2007; Morgan et al, 2008). To

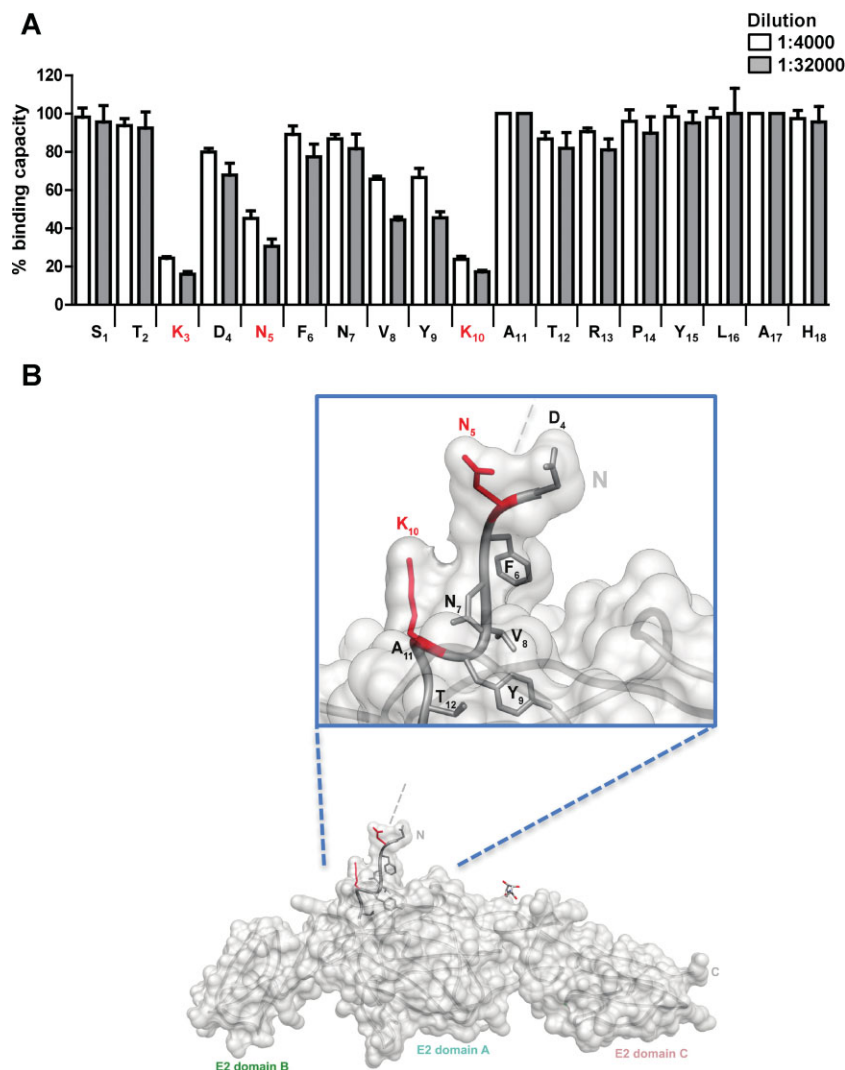


Figure 4. Alanine-scan analysis of E2EP3 by anti-CHIKV antibodies.

A. Alanine substitutions were constructed at each position of E2EP3 except the existing alanine residues. CHIKV-infected patients' plasma pools were used to validate the binding capacity. Plasma pools (Median 10 days post-infection) were tested in triplicates at dilutions from 1:2000 to 1:32,000 as indicated in Supporting information Fig 3. Shown are data representing different dilutions at 1:4000 and 1:32,000. Results are expressed as percentage binding capacity relative to the original E2EP3 sequence (% binding capacity) \pm SD. Experiments were performed in triplicates.

B. Schematic diagram showing the localization of the asparagine (N₅) and lysine (K₁₀) residues within the E2EP3 epitope region in the E2 glycoprotein based on structural data retrieved from PDB records: 3N44. The structure for K₃ was not resolved and therefore could not be localized. Tertiary structure of E2 glycoprotein is arranged into three structural domains (E2 domain A, amino terminal; E2 domain B, centre; E2 domain C, carboxyl terminal). Enlarged image shows the spatial position of the different amino acid residues within E2EP3 with N₅ and K₁₀ highlighted in red.

explore whether the E2EP3 epitope is also a main target for the protective response, plasma samples from CHIKV-infected NHP were characterized with regards to their reactivity against E2EP3. Nine days after CHIKV-infection, plasma samples had already detectable anti-CHIKV IgG titers and importantly, also detected E2EP3 specifically (Fig 7A and Supporting information Fig 2B). In *in vitro* neutralization assays CHIKV-infected NHPs plasma reduced CHIKV infectivity by 80% (Fig 7B). Addition of soluble E2EP3 peptide abrogated the inhibitory effect of monkey plasma samples significantly throughout the whole dilution series (from 1:100 to 1:3200) when compared to the untreated plasma samples (Fig 7B). Thus, as in humans, E2EP3 antibodies are part of the protective CHIKV response in NHPs.

The potential of E2EP3 epitope as a vaccine target was further assessed in a mouse model (Gardner et al, 2010). For this, C57BL/6 mice were vaccinated with E2EP3 covalently linked to KLH in the presence of Freund's Adjuvant. Mice were primed and boosted twice with the immunogen (emulsified first with Complete [CFA] and then with Incomplete Freund's Adjuvant [IFA]) over a period of 21 days. Significant anti-E2EP3 titer was detected 19 days post-vaccination after the 1st boost (Supporting

information Figs 2C and 5A) and was further increased after the 2nd boost at 27 days post-vaccination (Supporting information Fig 5B). Importantly, the sera obtained at 27 days post-vaccination were able to neutralize CHIKV-infection *in vitro*. Compared to the PBS-vaccinated control group, infectivity was reduced by approximately 40% (Fig 8A). Moreover, virus challenge in mice at 30 days post-vaccination indicated a partial protection by E2EP3 as viremia was reduced from 4500 to 2000 pfu/ml at 2 days post-challenge (Fig 8B). This reduction of virus titer was also reflected in clinical symptoms used to monitor the virus-induced inflammation (Supporting information Fig 5C). Maximal footpad swelling in the PBS-vaccinated group was more than twice as that of the E2EP3-vaccinated group (Fig 8C). E2EP3 may therefore be used both as a marker as well as a potential vaccine component in pre-clinical models for CHIKV therapy.

DISCUSSION

In a recent study on the naturally-acquired antibody response in CHIKV-infected patients, we reported that anti-CHIKV IgG can

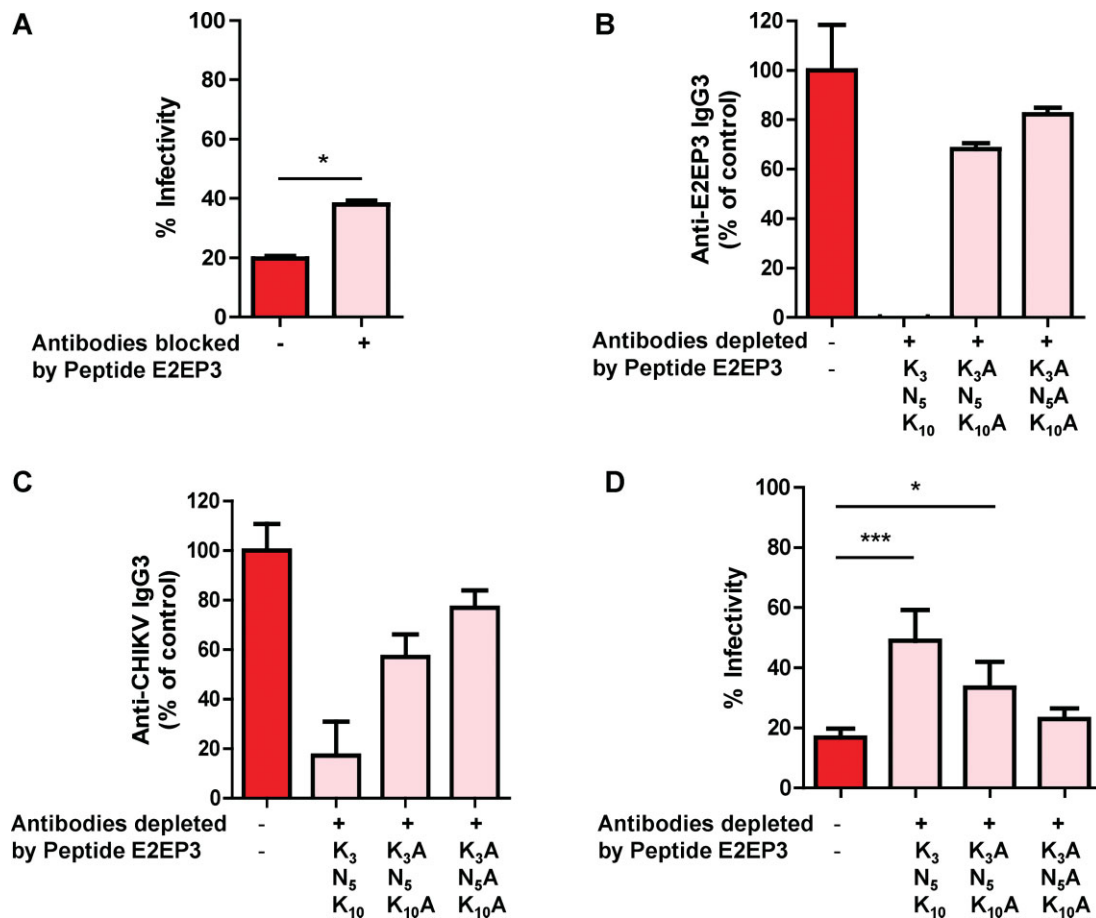


Figure 5. Depletion of anti-E2EP3 antibodies reduces neutralizing activity against CHIKV infection.

- A.** Anti-E2EP3 antibodies in patients' plasma pools were specifically blocked by soluble E2EP3 peptide and followed by *in vitro* neutralization assay as described in *Materials and Methods*. Results are expressed as percentage control infection. Data are presented as mean \pm SD. Neutralization assays were performed at 1:500 dilution ($n = 3$). * $p < 0.05$, Mann-Whitney U test.
- B.** Alanine substituted peptides did not deplete E2EP3-specific antibodies in pooled patients' plasma. Plasma samples (Median 10 days pio) were incubated with E2EP3 (K₃, N₅, K₁₀), E2EP3 with double alanine substitution at lysine residues (K₃A, N₅, K₁₀A) or triple alanine substitution at lysine and asparagine (K₃A, N₅A, K₁₀A) peptides. E2EP3 specific peptide-based ELISA was performed to measure the depletion efficiency. Results are expressed as percentage control IgG3 titer from non-depleted samples. Data are presented as mean \pm SD. Experiments were performed in triplicates.
- C.** Depleted samples as described in (B) were subjected to anti-CHIKV IgG3 antibodies detection. Virion-based ELISA was performed as described to measure the depletion efficiency. Results are expressed as percentage control IgG3 titer from non-depleted samples. Data are presented as mean \pm SD. Experiments were performed in triplicates.
- D.** *In vitro* neutralizing activity of anti-E2EP3 antibodies against CHIKV-infected patients' plasma samples. E2EP3 specific antibodies from pooled plasma samples (Median 10 days pio) were depleted by E2EP3 (K₃, N₅, K₁₀), E2EP3 with double alanine substitution (K₃A, N₅, K₁₀A) and triple alanine substitution (K₃A, N₅A, K₁₀A). Neutralization assays were performed at 1:500 dilution ($n = 3$). Non-depleted plasma and healthy plasma were used as controls. Results are expressed as percentage control infection. Data are presented as mean \pm SD. * $p < 0.05$; *** $p < 0.001$ by one-way ANOVA with Tukey post-test.

be detected only at the early convalescence phase of median 10 days pio (Kam et al, 2012). At this time point most of the virus has already been cleared and is usually no longer detectable in the blood. More surprisingly, we observed that virtually all anti-CHIKV IgG found at this stage of the disease seems to be of the IgG3 isotype (Kam et al, 2012). Moreover, IgG3 is one of the two antibody subclasses that can be readily transmitted across the placenta (Palmeira et al, 2012), further suggesting that protection of newborns from CHIKV infections can occur via maternal immunization (Englund, 2007; Gerardin et al, 2008). While it was expected that the early neutralizing antibody

response is targeting the proteins of the envelope of the virus, we have shown here now that in fact most of these IgG3 antibodies recognize a single epitope forming a prominently exposed stalk on the E2 glycoprotein.

When using complete CHIKV virion particles E2 glycoprotein was the only surface protein that reacted to the IgG of the patients' plasma collected during the early convalescent phase. We (Warter et al, 2011) and others have previously shown that other structural proteins including the E1 glycoprotein (Cho et al, 2008a; Kowalzik et al, 2008; Yap et al, 2010) and capsid (Cho et al, 2008b) are also detected to varying degrees by

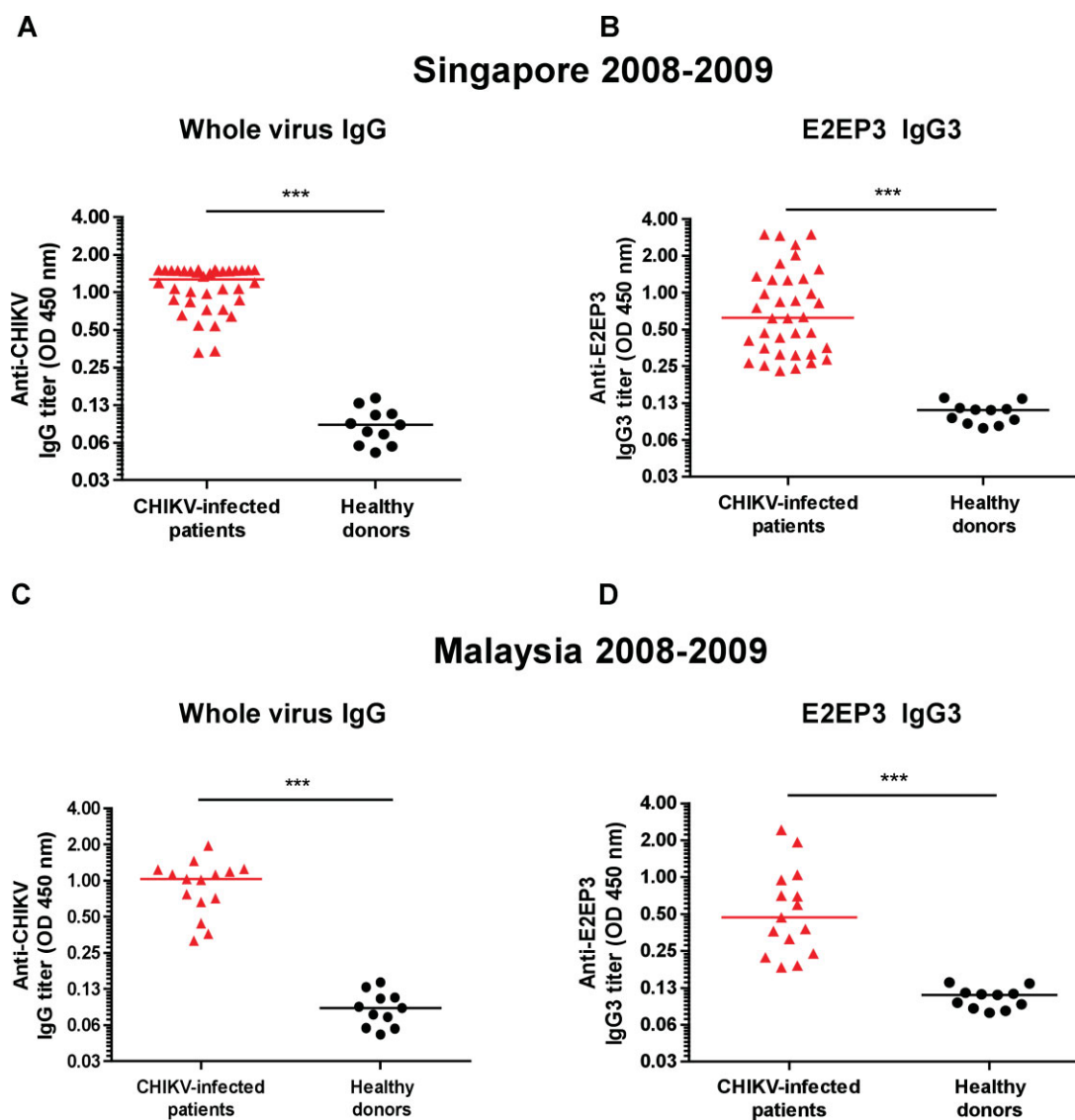


Figure 6. Sero-positivity of anti-E2EP3 IgG3 antibodies in CHIKV-infected patients from other cohorts.

- A.** CHIK virion-based ELISA was used to assess anti-CHIKV IgG titer in CHIKV-infected patients from another Singaporean cohort collected at median 10 days *pi*o ($n = 36$). Healthy donors' plasma ($n = 11$) were used as controls. Individual samples were subjected to virion-based ELISA at a dilution of 1:2000, followed by secondary human anti-IgG-HRP. *** $p < 0.001$ by Mann-Whitney U test. Experiments were performed in triplicates.
- B.** CHIKV-infected patients' and healthy donors' plasma were screened for IgG3 specific antibodies recognizing E2EP3 in the peptide-based ELISA. Individual samples were subjected to E2EP3 specific peptide-based ELISA at a dilution of 1:200, followed by secondary human anti-IgG3 isotype HRP. *** $p < 0.001$ by Mann-Whitney U test. Experiments were performed in triplicates.
- C.** CHIK virion-based ELISA were used to assess anti-CHIKV IgG titer in 15 CHIKV-infected patients from another cohort collected in Malaysia at median 14 days *pi*o. Healthy donors' plasma ($n = 11$) were used as controls. Individual samples were subjected to virion-based ELISA at a dilution of 1:2000, followed by secondary human anti-IgG-HRP. *** $p < 0.001$ by Mann-Whitney U test. Experiments were performed in triplicates.
- D.** CHIKV-infected patients' and healthy donors' plasma were screened for IgG3 specific antibodies recognizing E2EP3 in a peptide-based ELISA. Individual samples were subjected to E2EP3 specific peptide-based ELISA at a dilution of 1:200, followed by secondary human anti-IgG3 isotype HRP. *** $p < 0.001$ by Mann-Whitney U test. Experiments were performed in triplicates. The same set of healthy donors' plasma comprising of donors from Singapore and Malaysia were used as controls throughout the study. The y axis is plotted in log₂ scale. Red straight line represents the median of the CHIKV-infected patients' group and black straight line represents the median of the healthy donors' group.

patients' IgGs from patients' samples collected at later time points. However, especially at the early phase of infection the E2 glycoprotein is apparently the only major target. At later time points, contributions by epitopes of other proteins may further

increase the complexity of the patterns of antigenic recognition (Nowak, 1996). CHIKV represents a 'novel' virus for the naïve population. Most infected individuals did not have any prior encounters with CHIKV, and therefore lacked the complete

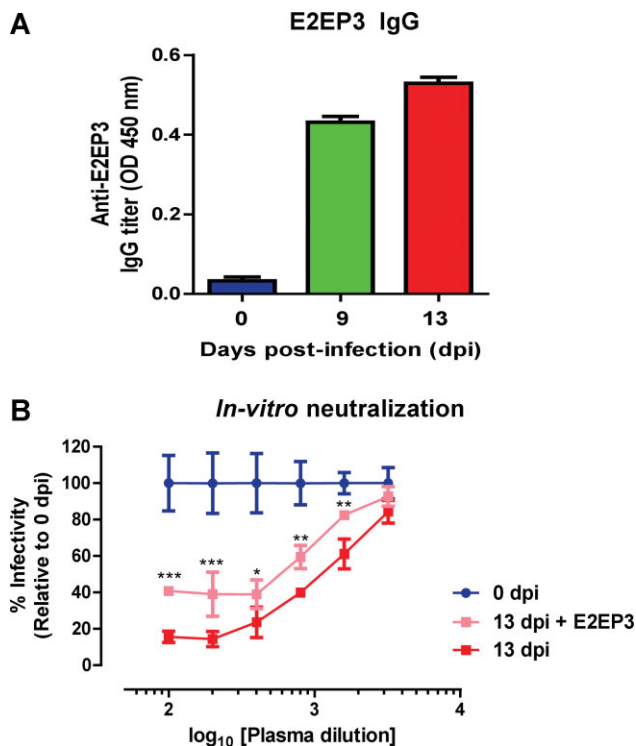


Figure 7. IgG from CHIKV-infected NHP plasma recognize E2EP3 and neutralize CHIKV infection *in-vitro*

- A.** E2EP3 specific antibodies titers in plasma samples (0, 9 and 13 days pi) were determined by E2EP3 specific peptide-based ELISA at a dilution of 1:2000. Data are presented as mean \pm SD.
- B.** Anti-E2EP3 antibodies in CHIKV-infected NHP plasma were specifically blocked by soluble E2EP3 peptide, and followed by *in vitro* neutralization assay as described in *Materials and Methods*. Results are expressed as percentage infection relative to 0 dpi. Data are presented as mean \pm SD. A set of serial dilutions from 1:100 to 1:3200 was made and samples assayed were performed in triplicates. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ by two-way ANOVA with Bonferroni's multiple comparisons test.

CHIKV-specific antibodies. E2EP3 may be an early target since it is a structural element shared with other alphaviruses (Ozden et al, 2008).

While E2 glycoprotein was clearly the dominant surface antigen, the most striking observation was that a vast majority of the early anti-CHIKV IgG3 antibodies were directed against a single linear epitope. Depletion experiments indicated that E2EP3-specific antibodies represented nearly 70 to 80% of the anti-CHIKV IgG of the patients' sera (Fig 5C). Published crystal structure data and alanine scan revealed the precise location of this dominant epitope. E2EP3 is located at the N-terminus of the E2 glycoprotein. It is part of the furin-loop and forms a prominent little stalk facing away from the virus envelope with sufficient flexibility for antibody recognition. While it almost appears to be 'destined' to be recognized by antibodies, its surface exposure is likely to be a consequence of the need to be reached by furin. Furin is a golgi-resident protease (Thomas, 2002) and is also used by various viruses including HIV (Hallenberger et al, 1992). It is mandatory for the maturation of alphaviruses where it facilitates cleavage of the p62 precursor

into E2 and E3 glycoproteins (Heidner et al, 1996; Ozden et al, 2008; Zhang et al, 2003).

We have shown that early anti-CHIKV IgG3 were strongly neutralizing (Kam et al, 2012). Here, we extended these findings verifying that E2EP3-specific antibodies are able to block viral infection (Fig 5). Experiments further showed that neutralizing antibodies to this epitope were also present in plasma samples of NHPs (Fig 7). Thus, E2EP3 is important for viral defence both in humans as well as in the pre-clinical animal model commonly used for the study of CHIKV infections. For CHIKV, many studies have used inactivated virus (Tiwari et al, 2009), subunit vaccine formulations (Akahata et al, 2010) or viral vectors (Wang et al, 2011) with some successes in pre-clinical models. Notably, E2EP3 is a true linear determinant. In mice, we could therefore show that short E2EP3 peptides linked to KLH are indeed able to induce protective antibody responses. E2EP3 therefore represents an ideal candidate that could be incorporated in vaccine formulations such as multiple antigenic peptides (MAPs), recombinant proteins or even virus-like particles (VLPs) aiming to prevent CHIKV infections. These formulations would have the advantage to incorporate CHIKV T helper epitopes for efficient antibody production. As a basic proof-of-principle, we showed in the mouse model that a simple peptide formulation was effective at inducing neutralizing antibodies that not only reduced viremia, but also diminished viral induced-pathologies such as joint inflammation (Gardner et al, 2010; Morrison et al, 2011) (Fig 8).

Antibodies to E2EP3 were detected during early convalescence after viremia was cleared. They serve as reliable early serologic markers for CHIKV infections. In three independent cohorts (2 from Singapore and 1 from Malaysia), E2EP3-specific antibodies were detected in almost all the blood samples taken between 10 and 14 median days pi from infected patients, whereas none of the control plasma reacted against the epitope. E2EP3 may therefore be used in diagnostic kits, such as epitope-based immunochromatographic tests (ICT) (Cuzzubbo et al, 2001; Marot-Leblond et al, 2009). In addition, E2EP3 could also be used for serology detection in sylvatic infections of primates just like screening of SIVs-infected animals with peptides in Africa (Simon et al, 2001; Worobey et al, 2010).

In summary, we established that the naturally-acquired early IgG3 response against CHIKV is strongly focused on the E2EP3 epitope. As a simple linear epitope, it may open new options for both diagnostic and prevention of CHIKV infections. Due to the resurgence of CHIKV and other alphaviruses, interests for prophylactic vaccines have already regained importance. Such vaccines would be useful for travellers and/or populations at risk during outbreaks and E2EP3 could become an integral component to achieve protection.

MATERIALS AND METHODS

Study subjects

Thirty patients who were admitted with acute CHIKF to the Communicable Disease Centre at Tan Tock Seng Hospital (CDC/TTSH), Singapore, during the outbreak from August 1 to September 23 2008

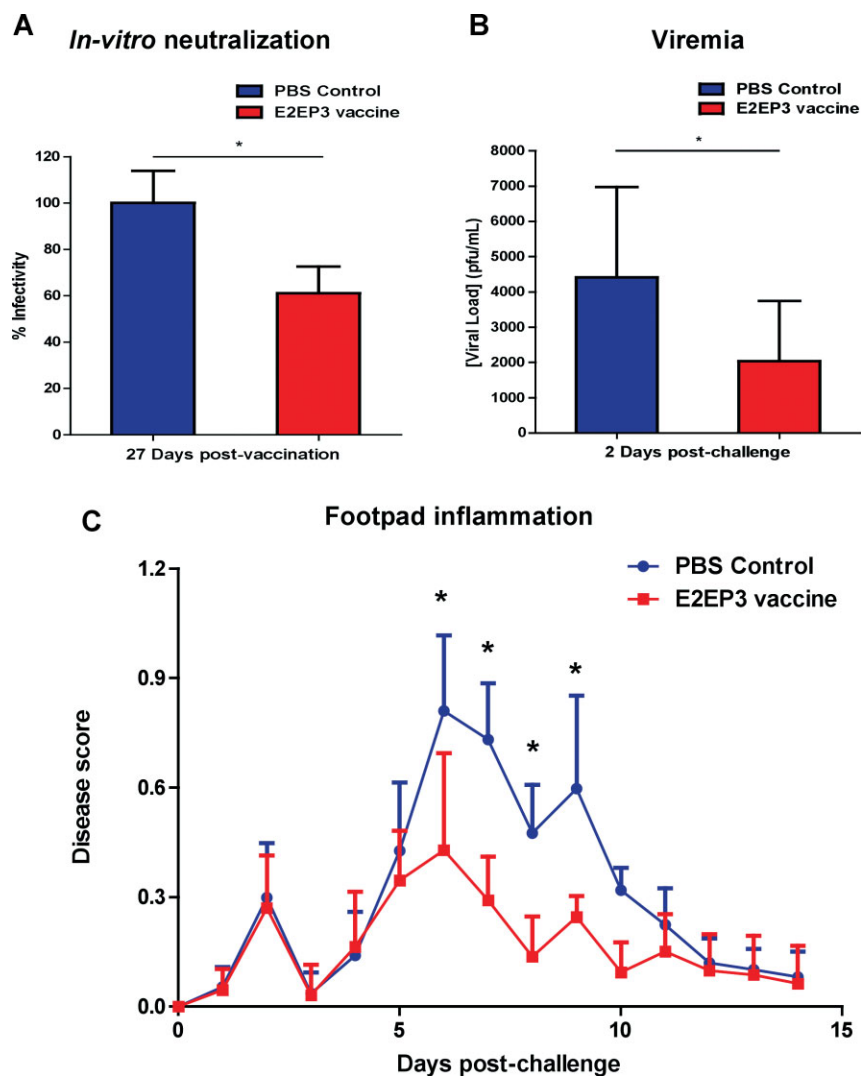


Figure 8. Protection against CHIKV challenge in mice vaccinated with E2EP3 peptide.

- A.** *In vitro* neutralizing activity of E2EP3-vaccinated mouse sera. Mice were immunized with E2EP3 peptide complex to KLH or PBS Control emulsified with Complete Freund's Adjuvant (CFA) subcutaneously, and were boosted two more times with Incomplete Freund's Adjuvant (IFA). Sera was collected at 27 days post-vaccination and assayed for *in vitro* neutralization at a dilution of 1:100 ($n = 3$). Results are expressed as percentage infection relative to PBS Control. Data are presented as mean \pm SD. * $p < 0.05$ by Mann-Whitney *U* test.
- B.** Mice immunized with E2EP3 or PBS Control were challenged subcutaneously with 10^6 PFU CHIKV (SGP11). CHIKV viremia was measured at 2 days post-challenge by virus plaque assay. The detection limit was 1000 pfu/ml. Data are presented as mean \pm SD. * $p < 0.05$ by Mann-Whitney *U* test.
- C.** Disease score measurement. Footpad sizes from day 0 to day 14 post-challenge were quantified by [width \times thickness]. Footpad swelling (inflammation) relative to day 0 was obtained with the formula: [(day x - day 0) \div day 0], where x represents footpad sizes from day 1 to day 14. Data are presented as mean \pm SD. * $p < 0.05$ by Mann-Whitney *U* test.

(Ng et al, 2009; Win et al, 2010), were included in this study. Written informed consent was obtained from all participants. This study was approved by the National Healthcare Group's Domain-specific Ethics Review Board (DSRB Reference No. B/08/026). Clinical features definition and clinical samples were as described previously (Ng et al, 2009; Win et al, 2010). Thirty-six other CHIKF patients were recruited from the same hospital and a single sample was taken during admission without further follow up. Serum samples were also obtained from fifteen CHIKF patients (median 14 days pio) seen at the University Malaya Medical Centre in Kuala Lumpur in 2008–2009.

Virion-based ELISA

The Singapore strain (SGP11) was isolated from a CHIKF patient (Her et al, 2010). Purified CHIKV (20,000 virions per μ l in PBS; 50 μ l per well) were immobilized on 96-well maxisorp microtitre plates (Nunc). Wells were blocked with PBS containing 0.05% Tween-20 and 5% non-fat milk (PBST-milk), and plates were incubated for 1.5 h at 37°C. CHIKV-infected human and NHP plasma samples were then diluted at 1:1000 to 1:2000 in PBST supplemented with 2.5% non-fat milk and incubated for 1 h at 37°C. HRP-conjugated goat anti-human IgG (Molecular Probes), mouse anti-human IgG3 (Molecular Probes), and

The paper explained

PROBLEM:

Chikungunya fever (CHIKF) is an acute illness with abrupt fever, skin rash, joint inflammation, and occasional involvement of the nervous system, heart and liver. It has emerged as an important viral disease in Asia and the Pacific region. It is caused by Chikungunya virus (CHIKV), an alphavirus that is transmitted by infected *Aedes* mosquitoes. There are no specific or effective treatments for CHIKF as there are no anti-virals or licensed vaccines. Patient management and treatment is largely symptomatic relief and primarily anti-inflammatory drugs during disease onset. The re-emergence of CHIKV has prompted interests to further characterize the understudied CHIKV and to devise strategies to halt viral transmission.

RESULTS:

During early disease onset, naturally-acquired anti-CHIKV antibodies from patients mostly target a linear dominant epitope

'E2EP3' located at the N-terminus of the E2 glycoprotein. Up to 80% of anti-CHIKV antibodies are specific against E2EP3 and removal of anti-E2EP3 antibodies partially reversed the neutralizing effects. Sero-positivity of E2EP3 validated across different patient cohorts confirmed that E2EP3 could be a good serology marker for early CHIKV detection. Moreover, anti-E2EP3 antibodies were also detected from CHIKV-infected non-human primates. Furthermore, mice vaccinated with E2EP3 peptides showed better clinical outcomes with reduced viremia and reduced joint inflammation.

IMPACT:

This study demonstrates that the early naturally-acquired antibody response is directed against a single epitope, and provides solid preclinical data that the linear E2EP3 epitope could play a role in future diagnostic and preventive applications.

rabbit anti-monkey IgG (Alpha Diagnostic) were used to detect human and NHP antibodies, respectively. Reactions were developed using TMB substrate (Sigma-Aldrich) and terminated by Stop reagent (Sigma-Aldrich). Absorbance was measured at 450 nm. Healthy donor samples were used as controls. ELISA readings were done in duplicates.

Epitope determination and structural localization

Peptide-based ELISA was performed to screen CHIKV-infected patients' plasma for viral epitopes using synthesized biotinylated-peptides (Mimotopes). Eighteen-mer overlapping peptides were generated from consensus sequence based on alignments of different CHIKV amino acid sequences (accession numbers: EF452493, EF027139, DQ443544, EU703760, EF012359, NC004162, FJ445430, FJ445431, FJ445432, FJ445433, FJ445463, FJ445502 and FJ445511). Synthesized biotinylated-peptides were dissolved in dimethyl sulphoxide (DMSO) to obtain a stock concentration of approximately 15 µg/ml. All the peptide samples were screened in triplicates using plasma from either CHIKV-infected patients or healthy donors, as well as in the absence of plasma in peptide-based ELISA assays. Structural data was retrieved from PDB (id: 3N44 and 2XFB) and visualized using the software CHIMERA (Pettersen et al, 2004). Solvent excluded molecular surfaces were generated with the help of MSMS package (Sanner et al, 1996). Coloring of the E2 domains and orientation of the E1–E2 heterodimer asymmetric unit relative to the viral membrane are based on previously described data (Voss et al, 2010).

Alanine scanning

Eighteen peptide sequences were synthesized with substitution of a native amino acid for an alanine (EMC microcollections GmbH). Peptides were dissolved in DMSO to obtain a stock concentration of approximately 15 µg/ml. All the peptide samples were screened in triplicates using plasma from either CHIKV-infected patients or

healthy donors. Results are expressed as percentage binding capacity relative to the original E2EP3 sequence peptide.

Affinity depletion of CHIKV anti-E2EP3 antibodies

For affinity depletion of human anti-E2EP3 antibodies, synthetic biotinylated E2EP3 peptide (EMC microcollections GmbH) was added at 450 ng/well to streptavidin-coated plates (Pierce) and incubated at room temperature for 1 h in PBS containing 0.1% Tween-20 (0.1% PBST). Human plasma samples were added and incubated for 25 min at room temperature for absorption. The unbound portion was collected after 21 rounds of absorption. ELISA analysis was performed to verify the levels of the antibodies during affinity depletion.

Peptide blocking assay

Synthetic soluble E2EP3 peptide (EMC microcollections GmbH) (100 µg/ml) was mixed with diluted (1:500) heat-inactivated human plasma or serially diluted (from 1:100 to 1:3200) heat-inactivated NHP plasma and incubated for 1 h at 37°C with gentle agitation (350 rpm). Samples were then mixed with CHIKV at Multiplicity of Infection (MOI) 10 and incubated for 2 h at 37°C with gentle agitation (350 rpm). Sero-neutralization assay was performed to verify the neutralizing activity.

Sero-neutralization assay

Neutralizing activity of antibodies from CHIKV-infected patient samples, NHP plasma and vaccinated mouse sera were tested in triplicates and analysed by immunofluorescence-based cell infection assay in HEK 293T cells. CHIKV was mixed at MOI 10 with diluted heat-inactivated human plasma, NHP plasma or vaccinated mouse sera, and incubated for 2 h at 37°C with gentle agitation (350 rpm). Virus-antibody mixtures were then added to HEK 293T cells seeded in 96-well plates (1.0×10^4 cells per well) and incubated for 1.5 h at 37°C. Medium was removed, and cells were replenished with DMEM

medium supplied with 5% FBS and incubated for 6 h at 37°C before fixation with 4% paraformaldehyde followed by immunofluorescence staining. Cells were permeabilized with PBS containing 0.2% Tween-20 and incubated for 10 min at room temperature. Cells were stained with mouse antibody recognizing CHIKV antigen (Her et al, 2010) diluted in PBS for 1 h at 37°C. This was followed by incubation with goat anti-mouse secondary antibody conjugated to fluorescein isothiocyanate (FITC) for 1 h at 37°C. Cell nuclei were labelled with DAPI. Images were acquired and analysed quantitatively by the Cellomics ArrayScan VTI HCS Reader. Percentage of infectivity was calculated according to the equation: % Infectivity = 100 × (% responder from sero-neutralization group/% responder from virus infection group).

Rhesus macaques studies

Five-year-old cynomolgus macaques (*Macaca fascicularis*) were imported from Mauritius. All animals were negative for SIV, Simian T-Lymphotropic Virus, Herpes B virus, filovirus, SRV-1, SRV-2, measles, dengue and CHIKV, and were maintained in a biosafety level 3 facility. Studies were approved by the regional animal care and use committee ('Comite Regional d'Ethique sur l' experimentation animale Ile de France Sud', Fontenay-aux-Roses, France), reference number: 07-012, in accordance with European directive 86/609/EEC. Animals were infected with 10⁶ PFU (in 1 ml PBS) LR2006-OPY1 CHIKV by intravenous (i.v.) inoculation, as described (Labadie et al, 2010). Animals were bled and observed daily for one week then twice a week to assess viral replication, inflammation and clinical signs of infection. No virus could be detected in plasma samples at 9 and 13 days post-inoculation.

Mouse studies and vaccination

Lyophilized KLH-E2EP3 peptide was dissolved in DMSO (Sigma-Aldrich) to a working concentration of 5 mg/ml. Three-weeks old, female, C57BL/6J ($n = 7$) were vaccinated subcutaneously in the abdominal flank with 100 µg of KLH-E2EP3 peptide prepared in 100 µl emulsion with 50% Complete Freund's Adjuvant (CFA) (Sigma-Aldrich) in PBS. Vaccinated mice were further boosted another two times at day 14 and day 21 with 50 µg of the peptide prepared in Incomplete Freund's Adjuvant (IFA) (Sigma-Aldrich). Control mice ($n = 7$) were vaccinated with PBS/CFA and PBS/IFA on first vaccination and subsequent booster shots, respectively. Sera were collected from all mice at day 19 and day 27 post-vaccination for downstream E2EP3 peptide-based ELISA. All protocols were approved by the Institutional Animal Care and Use Committee of the Agency for Science, Technology and Research (A*STAR), IACUC number: 080383. At day 30, C57BL/6J mice from E2EP3-vaccinated and PBS-control groups were inoculated with 10⁶ PFU (in 50 µl PBS) SGP11 CHIKV. Virus was inoculated in the subcutaneous (s.c.) region at the ventral side of the right hind footpad, towards the ankle. Viremia and degree of inflammation were monitored. Viremia analysis was performed for day 2 and day 6 by plaque assays. Hind footpads of mice were measured daily using a Vernier calliper from day 0 to day 14 post-infection. Measurements were done for the height (thickness) and the breadth of the foot and quantified as [height × breadth]. Degree of inflammation was expressed as relative increase in footpad size as compared to pre-infection with the following formula: [(day x - day 0) ÷ day 0] where x is the footpad measurements for each respective day post-infection.

Statistical analysis

Data are presented as mean ± standard error mean (SEM) or as mean ± standard deviation (SD). Differences in responses among groups at various time points and between groups and controls were analysed using appropriate tests (Mann-Whitney U test, Kruskal-Wallis with Dunn's post-test, One-way ANOVA with Tukey post-test, Two-way ANOVA with Bonferroni's multiple comparisons test). Statistics were performed with GraphPad Prism 5.04.

Author contributions

YWK, FML, THT, DS, WWLL, CLC performed immunological, virological, biochemical cell-based assays, and animal studies. PR led the NHP study and RLG provided the samples. SH and JKW performed structural localization maps. YWK, KHW, JCT, OR, LR and LFPN conceptualized the study. YWK, FML, THT, WWLL, SH, CLC, YFC, AC, ICS, YSL, RTPL, JCT, PR, KHW, OR, LR and LFPN analysed the data; YWK, JCT, PR, OR, LR, and LFPN wrote the manuscript. All authors read and approved of the manuscript.

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Supporting information is available at EMBO Molecular Medicine online.

The authors declare that they have no conflict of interest.

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