Mapping and Immunological Response of Immunodominant B and T cell Epitopes of E2 Glycoprotein of Chikungunya Virus

Abstract

Chikungunya virus (CHIKV) is a mosquito-borne Alphavirus and belongs to family Togaviridae. Debilitating arthralgia and myalgia are common symptoms of Chikungunya disease. This virus is sometimes fatal to humans, however there is no effective therapy available till now. In many studies envelope E2 protein of CHIKV has been exploited for diagnosis and vaccine therapy. Here we studied the immunodominancy of conformationally synthesized epitopes of E2 protein. Out of seventeen peptides, ten (E2P3, E2P5, E2P7, E2P8, E2P9, E2P10, E2P11, E2P13, E2P16 and E2P17) were proved to be major epitopes of E2 protein based on B and T cell response. When these peptides were immunized through intramuscular route encapsulated in PLGA microspheres with CpG ODN as an adjuvant, some sequences showed peak antibody levels ranging 1, 80,000 to 2, 20,000 in outbred and inbred (H-2^d) strains of mice with memory response and they showed IgG2a and IgG2b subclass distribution. Depending upon their nature E2P1, E2P8, E2P10, E2P17 peptides showed high stimulation index during in vitro T cell proliferation assay. Plaque reduction neutralization test (PRNT) assay proved immunodominance of few epitopes showing strong neutralization of Chikungunya virus. Thus ten peptides were considered as B cell epitopes. On the basis of cell mediated immune response and cytokine profile four peptides were considered as T cell epitopes. Three peptides E2P8, E2P10, E2P17 showed common properties mediated immune response and cytokine profile four peptides were considered as T cell epitopes. Three peptides E2P8, E2P10, E2P17 showed common properties of B and T cells. These B and T cell epitopes can be assembled together in multiple antigenic peptides (MAPs) for developing effective immunogen for CHIKV.

Keywords: E2 protein; Peptides; B & T cell epitopes; Chikungunya

Abbreviations: CHIKV: Chikungunya Virus; TFA: Trifluoroacetic Acid; FCA: Freund's Complete Adjuvant; BCA: Bicinchoninic Acid; MAPs: Multiple Antigenic Peptides

Introduction

Chikungunya virus (CHIKV) belongs to a group of mosquito borne Alphaviruses [1]. This virus is transmitted to humans by Aedes mosquitoes. Chikungunya disease was reported for the first time in East Africa in 1952 [2,3] and distributed to many geographical areas. It was due to adaption of CHIKV to a widely distributed novel vector Aedes albopictus [4-6]. CHIKV infection is generally associated with fever, headache, photophobia, myalgia and arthralgia [7,8]. The acute phase of CHIKV infection typically lasts from days to weeks however joint pain and chronic fatigue can persist upto months to years [9-11]. CHIKV have positive-sense RNA genome, encodes four non-structural (nsp1-4) and five structural (Nucleocapsid, E1, E2, E3, 6K) proteins. The E1 glycoprotein participates in cell fusion [12] while the E2 glycoprotein binds to cellular receptor [13] and initiate endocytosis [14]. We focused our attention on E2 glycoprotein because of its involvement in host-cell receptor interaction and exposed on viral surface [15] which can be the target of virus neutralization. We selected and synthesized seventeen peptides of E2 protein (S27 CHIKV strain) to map the B and T cell epitopes. Ten peptides showed B cell response during competitive immunoassay direct binding assay, high antibody titer with memory response and four peptides showed T cell response during lymphoproliferative assay and cytokine profile. These three peptides have overlapping B and T cell response. Neutralization assay of peptide specific antibodies proved substantial number of neutralizing epitopes on E2 protein. This study may help to design an immunogen for CHIKV.

Materials and Methods

Selection and chemically synthesis of peptides of E2 protein

Peptides were selected using DNASTAR (on the basis of hydrophilicity, hydrophobicity, secondary structure, antigenicity index, amphipathicity) and TEPITOPE software (binding time(T1/2) of peptides to MHC molecules), 17 peptide sequences spanning entire E2 protein (CHIKV S-27 strain) were selected (Table 1) and synthesized by solid phase synthesis using Fmoc chemistry. Peptides were cleaved using trifluoroacetic acid (TFA) containing scavengers as anisole and thioanisole. Purification of peptides was done initially by Gel filtration and later by HPLC using C18 column. After purification, peptides were authenticated by physical-chemical method like amino acid analysis and sequencing. On the basis of chemical nature all the peptides were dissolved in a proper solvent. Acidic peptides were dissolved in DMSO while basic peptides were dissolved in PBS solution (pH 7.0 to 7.4). Basic peptides were dissolved in PBS with optimum amount of trifluoroacetic acid and neutral peptides were dissolved in DMSO with PBS.
Sequences
PVIGREKFHSRPQHGGKELP
2855-2868
SMGEEPNYQEEWVTHK
GFTDSRKISHSCTHPFHHD
2865-2880
LSANGTAHGHPHE
MVPKARNPTVTYGKNQ
3066-3081
IGTDDSHDWTKLRY
KLRYMDNHIPADAGRA
2933-2952
2913-2931
2954-2972
DTPDRTLLSQQSGNVKIT
3013-3032
NSPLVPRNAELGDRKGKI
2800-2820
2991-3008
2881-2896
STKDNFNVYKATRPYLAHCPD
2973-2989

Table 1: Peptide sequences selected on the basis of DNASTAR and TEPITOPE software.

| Peptides | Sequences | Sequence No. |
|----------|-----------|--------------|
| E2P1     | HCPDGEGHSCHSPV | 2817-2831 |
| E2P2     | RNIRNATDGTKLK | 2835-2847 |
| E2P3     | IGTDSHDWTKLRY | 2855-2868 |
| E2P4     | VNGRTVYKNCWGSNLEG | 2991-3008 |
| E2P5     | SMGEPRYQEEMWTHK | 3098-3113 |
| E2P6     | KLRYMDNHPADAGRA | 2865-2880 |
| E2P7     | GFYDSKIHSHCTHFHHD | 2913-2931 |
| E2P8     | PVIGREKFSRPQHGKELP | 2933-2952 |
| E2P9     | TYQVCGTAEEIEVHMP | 2954-2972 |
| E2P10    | DTPDRTLLSQQSGNVKIT | 2973-2989 |
| E2P11    | DKVINCKVDQCHAVTNHK | 3013-3032 |
| E2P12    | VPTEGELYTVGANEPY | 3120-3234 |
| E2P13    | LSANGTAHGHPHE | 3141-3153 |
| E2P14    | GLFVRTSACTTGTM | 2881-2896 |
| E2P15    | NSPLVPRNAELGDRKGKI | 3037-3054 |
| E2P16    | MYPKARNPTVYGKNO | 3066-3081 |
| E2P17    | STKDNFNVYKATRPYALCPD | 2800-2820 |

Mice

Six to eight weeks old outbred mice were procured from Experimental Animal Facility, AIIMS, New Delhi, India. BALB/c (H-2b) mice were procured from National Institute of Nutrition, Hyderabad, India. Each experimental group consisted of six to eight mice. All the animals were provided food and water in pathogen free condition. All experiments were conducted in accordance with the guidelines of AIIMS ethics as well as CPCSEA, Ministry of Social Justice, Government of India.

Microspheres preparation, size and morphology

Microspheres with entrapped peptides were prepared using poly (DL-lactide-co-glycolide; 50:50) (Sigma) by double solvent evaporation method (water-in-oil-in-water) [16]. The percentage entrapment (58-65%) for peptides was calculated by bicinchoninic acid (BCA) method. The size distribution (2-8µm) of the microspheres was determined by laser diffraction (Malvern Instrument, UK). Microspheres morphology was studied by scanning electron microscopy (Phillips, CM 10) and found to be spherical in nature.

Immunization and sera collection

Mice were immunized through Intramuscular route using, PLGA microspheres containing 40µg of peptide equivalent was suspended in 40 µl of PBS with 5µg of CpG ODN 1826 (TCCATGAGGTTCCGTACGTT) (Coley Pharmaceuticals, USA) and injected on the thigh region on day 0. Booster dose of 20µg of peptide with 5µg of CpG was given on days 23 and 42. In case of E2 protein, primary dose of 30µg in Freund’s complete adjuvant (FCA) was used on day 0 followed by booster dose of 15µg in incomplete Freund’s adjuvant (IFA) was given on day 23 and 42. Bleeds were collected from retro-orbital plexus on the day 0, 28, 42, 60, 90, 120. Pre immune sera were collected. Antisera was separated and stored at -20°C till use.

Competitive immunoassay

Briefly, varying amounts of peptides (0.125-256µg) were incubated with appropriate dilution of E2 protein antisera (Outbred mice antisera1: 16,000 dilutions, absorbance 1.0) for 2 h at 37°C (Full-length E2 protein was provided by Dr. M.M.Parida, DRDE, Gwalior, INDIA). This Ag-Ab complex was transferred onto the ELISA plate previously coated with E2 protein (100ng/well) and blocked with 5% milk powder in PBS. After washing with 0.05% PBS Tween-20, goat anti mouse IgG-HRPO conjugate (1:1000 dilution) was added and incubated for 1 h at 37°C. The color was developed using 0-phenylene diamine (OPD) as a chromogen and H2O2, as a substrate and absorbance was recorded at 492nm. The amount of peptide required for 50% inhibition with E2 protein was calculated. The results were expressed as the percentage of antibody binding in presence of competitor as compared to the control value (without competitor).

Humoral response (Antibody peak titer) of the peptides by ELISA

The peptide specific antibody levels were estimated in terms of peak antibody titer using standard ELISA protocol. Briefly microtiter plates (Immulon IIHB, Dynatech) were coated with 200 ng/well of each peptide (100 µl/well) in coating buffer and kept overnight at 4°C. After blocking, serial two-fold dilution of mice antisera from different groups were added and incubated at 37°C for 2 h, color was developed as described above and absorbance was taken at 492 nm. Empty microspheres and CpG antisera were used as negative control. The titers were expressed as the highest serum dilution giving an absorbance higher than that of empty microspheres (EM) + CpG.

Direct binding assay

Immunoreactivity of E2 protein antisera with different peptides were studied by direct binding assay. The ELISA plates were coated with different peptides (200ng/well). Conversely reactivity of each peptides antisera with E2 protein was also performed while plates were coated with E2 protein (100ng/well) and incubated with individual peptide. After blocking the plates, E2 antisera/peptide antisera was added at 1: 200 dilutions and incubated at 37°C for 2 h. Color was developed as described above

Estimation of IgG subclasses

IgG subclass (IgG1, IgG2a and IgG2b) estimation was done as per manufacturer’s instructions using isotyping kit ISO-2 (Sigma, USA).

Evaluation of B cell memory response

Mice were immunized with 20µg of native E2 protein with 5µg of CpG ODN on day 120 and bleeds were collected on day 135 and IgG antibody titers were performed as described above.
T cell proliferation assay

Outbred and inbred H-2d mice were immunized with 40µg of peptide in microspheres on day 0 containing 5µg of CpG ODN. Booster dose of 20µg was given on day 10. In case of E2 protein, primary dose of 30µg was given on day 0 followed by booster dose of 15µg on day 10. Mice were sacrificed on day 21. Single cell suspension of pooled splenocytes (devoid of B cells by panning with anti-mouse immunoglobulin) were cultured in triplicate wells and plated at 2 x 10^5 cells/well in RPMI 1640 medium (300µl/well) supplemented with gentamycin, streptomycin (50 µg/ml) and 10% fetal calf serum (FCS). Splenocytes were stimulated in vitro with different variables as described below.

A. Mice were primed with E2 protein and splenocytes in vitro stimulated with E2 protein (5, 10 and 25 µg/ml) or individual peptides (10, 25 and 50µg/ml).

B. Mice were primed with individual peptides and splenocytes in vitro stimulated with E2 protein and cognate peptide with same concentration as above.

PHA (2 µg/well) was used as positive control. 200µl/well of culture supernatant was collected after 72hrs and stored at -70°C for cytokine estimation. The cultures were pulsed with [3H] thymidine (specific activity 6.4 mmol-1, BARC, India) at 0.5µCi/well. The cells were harvested after 18hrs and thymidine incorporation was measured by β scintillation counter. Stimulation index was calculated by count per minutes (CPM) with stimulus divided by CPM without stimulus. The data were presented as a mean SI±SD of triplicate wells.

Cytokine level measurement

The levels of IL2, IL-4, IL-10, IL-17 IFN-γ cytokines were measured in the culture supernatant of duplicate wells by sandwich ELISA as per manufacturer’s instructions (e-Biosciences, USA).

Neutralization assays

90% plaque reduction neutralization test (PRNT90) was used for measuring neutralization by antibodies. Two-fold Serial dilutions of peptide specific antisera were incubated with 200 PFU of CHIKV (DRDE-06) for 90 min at 37°C. Antisera and virus complexes (200µl/well) were transferred onto 24 well culture plates (Corning NY) containing monolayer of Vero cells. Plate was incubated at a 37°C and rocked for every 20 min. Each well was overlaid with a 0.4% gene pure LE agarose/ DMEM medium layer. The agarose layer was removed after 48hrs then fixative/staining solution (40% methanol and 0.25 % crystal violet) were added for five minutes and rinsed with DDW. The neutralizing antibody titer was expressed as a reciprocal of dilution of antisera. The plaque number in control. Preimmune sera were used as a control.

Immunoreactivity of E2 protein antisera with peptides

Different peptide reactivity with E2 antisera was done by direct binding assay. Peptides E2P3, E2P5, E2P7, E2P8, E2P9, E2P10, E2P11, E2P13, E2P16 and E2P17 showed maximal immunoreactivity with E2 antisera at a fixed dilution (Figure 2A). However other peptides did not show significant reactivity with antisera indicating that E2 protein induced polyclonal antibodies for specific epitopes only (data not shown). The reactivity was highest with sera collected on day 60. In conclusion those peptides that showed competition immunoassay also showed direct binding with E2 antisera.

Epitope-identification using peptide-based ELISA

Individual peptide immunogenicity was tested in outbred and inbred mice. Peptides E2P3, E2P5, E2P7, E2P8, E2P9, E2P10, E2P11, E2P13, E2P16 and E2P17 showed high peptide specific IgG levels in all the bleeds. These peptides showed significantly high (p < 0.001) anti-body titers ranging from 1,80,000 to 2,20,000 in outbred strain on day 60 (Figure 2C). Antibody peak

Results

Competition immunoassay of E2 protein antisera with peptides

For competitive ELISA, the dilution of E2 protein antisera, that gave an absorbance of 1.0 was used and incubated with increasing amounts of different peptides. Among the individual peptides competed with E2 antisera, peptides E2P3, E2P5, E2P7, E2P8, E2P9, E2P10, E2P11, E2P13, E2P16 and E2P17 showed competition in a dose dependent manner (8µg-40µg) (Figure 1). Other peptides did not show competition even at higher concentration of competitor (Figure 1A&B). However, under similar experimental conditions, 2.5 µg of E2 showed 50% inhibition with its own anti-sera. Thus E2P3, E2P5, E2P7, E2P8, E2P9, E2P10, E2P11, E2P13, E2P16 and E2P17 appear to be potential B cell epitopes of E2 protein.
titers gradually increased from day 28 to 60 and later declined on day 120. Interestingly 135 day sera also showed booster effect with peak titers equal to 60 day sera after immunization with E2 protein. Immunogenicity of peptides was also verified in H-2d mice and showed slightly higher antibody titer as compared to outbred mice (Figure S1(C&D)). Rest seven peptides showed low antibody titers in both strains of mice. Peak titer of E2P1 and E2P6 peptides have shown in Figure 2C while other data not shown.

Immunoreactivity of peptides anti-sera with native E2 protein

Peptide anti-sera raised in outbred and inbred H-2d mice was evaluated for its reactivity with E2 protein. The sera raised for each of the peptides E2P3, E2P5, E2P7, E2P8, E2P9, E2P10, E2P11, E2P12, E2P16 and E2P17 had show differential immunoreactivity with E2 protein in both strains of mice (Figure 2B & S1(A&B)). Peptide’s anti-sera collected at day 135 also showed significant reactivity (P<0.001) with E2 protein. Immunoreactivity of peptides antisera with native E2 proved the presence of epitopes on surface of E2 protein.

B-cell Memory response

Memory response on day 135 through generation of high antibody levels were observed with all ten peptides in outbred and inbred mice Figure 2C & S1 (C&D). It indicates that ten peptides had the potential to induce the secondary immune response after challenge with E2 protein.

IgG subclass estimation

Isotyping of all ten peptides antisera revealed some interesting

![Figure 2(A): Direct binding assay of peptides with E2 protein antisera, 200ng/100µl peptides were coated onto ELISA plates and E2 protein antisera were added at 1:200 dilutions, color was developed and absorbance was taken at 492 nm. Preimmune sera (PIS) was used as negative control. Reactivity was checked by using ELISA protocol. Empty microspheres (EM) + CpG ODN were taken as negative control. Data of two independent experiments expressed as Mean ± SD. ***P< 0.001.](image1)

![Figure S1(A&B): Direct binding assay of peptides antisera with E2 protein raised in H-2d mice Reactivity was checked by using ELISA protocol. Empty microsphere (MS) + CpG ODN was taken as negative control. Data of two independent experiments expressed as mean ±SD. ***P< 0.001.](image2)

![Figure 3: Estimation of Peptide specific IgG isotype antibodies (IgG1, IgG2a and IgG2b) in outbred mice on days 42, 60, 90 and 135. ELISA plates were coated with peptides and peptides antisera were added at fixed 1:200 dilutions. HRP-conjugated goat antimouse-IgG1, IgG2a and IgG2b were used. Experiments were done twice and data was represented as mean absorbance of pooled antisera.](image3)
results. In case of outbred mice, IgG2a/IgG2b antibody levels were significantly higher (P<0.005) for E2P3, E2P11, E2P16 followed by E2P7, E2P13 (p=0.05) peptides (Figure 3). Rest of the peptides E2P5, E2P8, E2P9, E2P10, and E2P17 showed equal distribution of IgG1 and IgG2a/IgG2b subtypes indicating mixed Th1 & Th2 biased immune response. Comparable results were obtained with inbred strain (Figure S2) indicating isotyping antibody level is not strain restricted.

**T cell proliferation assay**

After stimulation of E2 protein primed splenocytes with individual peptides or E2, of seventeen peptides only four peptides E2P1, E2P8, E2P10 and E2P17 showed significantly high (P<0.001) stimulation index (SI) as compared to other peptides. Peptides E2P1, E2P8, E2P10 showed stimulation index 36±4.89 to 40±5.12 at 25µg/well while E2P17 showed 22±3.45 at 50µg/well. Stimulation with E2 protein showed similar SI value at 5µg/well (Figure 4A). Peptide primed splenocytes after stimulation with cognate peptides or E2 protein again E2P1, E2P8, E2P10, E2P17 showed the highest SI value (Figure 4B). These results proved that four dominant T cell epitopes are presents on E2 protein. Same peptides consistently showed high SI value in inbred mice too (Figure S3).

**Estimation of Th1, Th2 and Th17 cytokines level**

Cytokine levels were measured in the culture supernatant of

a. E2 protein primed splenocytes stimulated with E2 protein and peptides

b. Peptides primed splenocytes stimulated with cognate peptides and E2 protein in outbred and inbred mice.

E2 protein primed splenocytes of outbred mice stimulated with peptides showed the following results. E2P1, E2P8, E2P10 and E2P17 peptides produced significantly high IFN-γ (1207-1560 pg/ml, p < 0.005) as compared to rest of the peptides (220-546 pg/ml). Of interest IL-2 level was significantly higher (180-240pg/ml, P<0.001) with peptides E2P1, E2P8, E2P10 and E2P17 as compared to other peptides (28-98 pg/ml). IL-4 level was insignificant for all the peptides. Interestingly only E2 protein and its E2P5 peptide produced IL-10 ranging 145-185 pg/ml and its levels were insignificant for rest of the peptides (32-59 pg/ml). Significantly high level of IL-17 (302-380 pg/ml, P=0.001) was observed after stimulation with E2P1, E2P8, E2P10, E2P17 peptides (Table 2).

In another formulation peptides primed splenocytes when in vitro stimulated with cognate peptides again E2P1, E2P8, E2P10, E2P17 peptides showed IFN-γ level in the range 1518-1729 pg/ml compared to other peptides (200-400pg/ml). Rest cytokine levels were in same pattern as of E2 protein primed splenocytes shown in Table 2. Similar cytokine profiles were observed in case of inbred mice as shown in supplementary data Table S1 &S2. Finally results conclude that peptides E2P1, E2P8, E2P10 and E2P17 produced significant levels of IL-2, IL-17, IFN-γ cytokines.
# Table 2: Cytokine level (pg/ml) in culture supernatant of splenocytes of Outbred mice.

| Peptides | IL-2       | IL-4       | IL-10      | IL-17      | IFN-γ     | B       |
|----------|------------|------------|------------|------------|-----------|---------|
| E2P1     | 240 ± 6.16 | 68 ± 5.67  | 42 ± 0.12  | 380 ± 6.04 | 1489 ± 4.02 | A       |
| E2P2     | 40 ± 2.12  | 54 ± 3.29  | 48 ± 7.35  | 92 ± 5.98  | 234 ± 5.12 |         |
| E2P3     | 81 ± 4.16  | 62 ± 6.24  | 28 ± 9.27  | 56 ± 6.45  | 200 ± 0.32 |         |
| E2P4     | 39 ± 5.13  | 50 ± 5.25  | 32 ± 12.63 | 71 ± 7.24  | 212 ± 7.16 |         |
| E2P5     | 102 ± 3.05 | 77 ± 0.63  | 145 ± 3.30 | 125 ± 9.56 | 400 ± 6.45 |         |
| E2P6     | 25 ± 2.89  | 56 ± 5.28  | 38 ± 9.39  | 65 ± 7.65  | 123 ± 5.43 |         |
| E2P7     | 79 ± 4.35  | 59 ± 4.18  | 45 ± 6.02  | 56 ± 0.09  | 202 ± 4.12 |         |
| E2P8     | 190 ± 5.64 | 69 ± 6.39  | 38 ± 4.35  | 308 ± 8.87 | 1207 ± 14.2|         |
| E2P9     | 56 ± 8.00  | 79 ± 3.28  | 42 ± 12.73 | 87 ± 9.54  | 123 ± 5.02 |         |
| E2P10    | 202 ± 4.45 | 72 ± 3.26  | 39 ± 2.63  | 104 ± 7.73 | 367 ± 4.29 |         |
| E2P11    | 78 ± 7.08  | 56 ± 4.16  | 69 ± 6.30  | 101 ± 5.89 | 145 ± 7.24 |         |
| E2P12    | 76 ± 9.21  | 58 ± 5.27  | 32 ± 4.47  | 116 ± 6.54 | 234 ± 7.12 |         |
| E2P13    | 67 ± 5.12  | 52 ± 0.46  | 38 ± 6.56  | 124 ± 507  | 1589 ±12.14|         |
| E2P14    | 26 ± 0.21  | 56 ± 6.23  | 30 ± 7.92  | 116 ± 6.54 | 234 ± 7.12 |         |
| E2P15    | 38 ± 6.38  | 55 ± 8.35  | 47 ± 0.63  | 101 ± 4.63 | 415 ± 6.25 |         |
| E2P16    | 98 ± 7.42  | 70 ± 6.26  | 29 ± 5.02  | 97 ± 6.42  | 406 ± 8.16 |         |
| E2P17    | 180 ± 21.45| 40 ± 5.25  | 79 ± 6.08  | 302 ± 0.39 | 1408 ± 9.34|         |
| E2 protein| 222 ± 9.67 | 139 ± 6.24 | 185 ± 9.48 | 396 ± 6.26 | 1589 ±12.14|         |

A

E2 protein primed splenocytes stimulated with E2 protein and individual peptides, B Individual peptide primed splenocytes stimulated with individual peptides, 1EM–Empty microspheres. Data are representative of two independent experiments are shown as Mean ± SD.
Table S1: Cytokine level (pg/ml) in culture supernatant of E2 protein primed splenocytes of inbred mice and in vitro stimulated with E2 protein and peptides.

| Peptides | IL-2   | IL-4   | IL-10  | IL-17  | IFN-γ  |
|----------|--------|--------|--------|--------|--------|
| E2P1     | 231±5.26 | 62±1.37 | 28±4.23 | 349±7.19 | 1582±8.13 |
| E2P2     | 50±7.40  | 54±1.13 | 49±6.56 | 84±6.45 | 234±6.27  |
| E2P3     | 82±6.19  | 41±5.35 | 32±8.96 | 56±9.09 | 212±8.56  |
| E2P4     | 59±6.23  | 55±6.68 | 45±10.04| 48±8.87 | 245±9.89  |
| E2P5     | 100±8.42 | 61±8.09 | 176±6.9 | 108±9.65| 45±5.46   |
| E2P6     | 35±2.38  | 42±7.76 | 49±5.63 | 108±6.46| 101±6.12  |
| E2P7     | 79±3.67  | 49±9.68 | 38±4.69 | 76±0.97 | 322±5.07  |
| E2P8     | 200±7.19 | 69±7.76 | 28±5.36 | 321±6.54| 1325±1.254|
| E2P9     | 82±8.08  | 82±5.50 | 34±9.96 | 78±5.09 | 136±5.52  |
| E2P10    | 191±9.34 | 43±6.85 | 48±6.54 | 343±7.24| 1567±7.84 |
| E2P11    | 80±12.46 | 49±9.66 | 49±0.45 | 92±10.56| 381±5.95  |
| E2P12    | 28±2.4   | 45±0.85 | 88±7.69 | 46±6.84 | 166±7.84  |
| E2P13    | 77±6.23  | 48±7.49 | 44±3.54 | 106±8.93| 345±7.76  |
| E2P14    | 21±10.56 | 56±6.63 | 40±7.25 | 42±6.75 | 283±6.91  |
| E2P15    | 32±4.06  | 39±6.97 | 51±6.52 | 49±7.08 | 44±5.32   |
| E2P16    | 70±6.34  | 50±9.48 | 52±1.53 | 112±8.84| 448±5.64  |
| E2P17    | 165±8.45 | 45±10.07| 61±4.50 | 340±9.05| 1490±7.62 |
| E2 protein| 202±0.98 | 142±8.45| 214±2.00| 382±6.81| 1622±6.54 |
| 1EM      | 34±4.05  | 48±4.47 | 38±5.45 | 42±7.42 | 189±6.89  |

Data are representative of two independent experiments and are shown as Mean ± SD.

1. EM – Empty microspheres.

Table S2: Cytokine level (pg/ml) in culture supernatant of individual peptide primed splenocytes of inbred mice and in vitro stimulated with individual peptides.

| Peptides | IL-2   | IL-4   | IL-10  | IL-17  | IFN-γ  |
|----------|--------|--------|--------|--------|--------|
| E2P1     | 218±6.32 | 74±6.36 | 50±6.45 | 360±6.45| 1749±8.64 |
| E2P2     | 38±0.39  | 52±7.20 | 43±4.10 | 40±3.46 | 136±5.45 |
| E2P3     | 94±6.13  | 42±6.59 | 34±5.29 | 46±6.48 | 123±5.34 |
| E2P4     | 23±6.32  | 57±6.69 | 44±2.20 | 51±4.65 | 118±6.45 |
| E2P5     | 98±5.45  | 64±10.43| 176±4.19| 75±6.87 | 223±8.34 |
| E2P6     | 27±8.75  | 39±9.30 | 34±3.0 | 45±5.09 | 177±8.87 |
| E2P7     | 63±5.68  | 42±8.47 | 38±2.47 | 66±6.52 | 208±5.56 |
| E2P8     | 210±5.45 | 82±6.39 | 45±3.91 | 325±8.42| 1204±1.686|
| E2P9     | 56±6.00  | 62±6.92 | 46±2.16 | 76±6.92 | 184±0.23 |
| E2P10    | 199±0.21 | 35±6.46 | 43±6.20 | 305±13.75| 1590±14.10|

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Data are representative of two independent experiments and are shown as Mean ± SD.

1. EM – Empty microspheres.

**In vitro neutralization assay**

Peptides E2P3, E2P5, E2P7, E2P8, E2P9, E2P10, E2P11, E2P13, E2P16 and E2P17 antisera of day 60 was used for in vitro neutralization (PRNT90) assay. Antisera of E2P10 at 1:3200 dil, E2P3, E2P5, E2P17 at 1:800 dil, E2P7, E2P8, E2P13 at 1:200 dil and E2P16 at 1:100 dilution showed 90% plaque number reduction (Figure 5). However, E2P9 and E2P11 peptide antisera not showed significant neutralization for CHIKV.

**Discussion**

This study describes the mapping of Immunodominant B- and T- cell epitopes on envelope E2 protein of chikungunya virus. This study also describes the humoral and cellular responses of these synthetic epitopes entrapped in PLGA microsphere when immunized through intramuscular routes in murine model. Earlier reports indicate that formalin inactivated, attenuated, VLP based vaccine showed protection after CHIKV challenge in mice and macaques model through generation of neutralizing antibodies [17-20]. These studies though showed encouraging results but none of these have entered as commercial vaccine. In the case of CHIKV, B cell response is essential because monoclonal antibody against the specific epitope of E2 protein protected mice or inhibit virus effect on cell lines after CHIKV challenging [21-23]. All these studies mainly focused on antibody response but in our study we identified T cell epitopes of E2 protein that are involve in the effector functions of the immune response. In the present study determinants of E2 protein were identified by competitive immunoassay and direct binding assay. In Competitive immunoassay ten out of seventeen competed with E2 protein antibodies. Again reactivity of same peptides with E2 protein antisera clearly indicates that E2 antisera have polyclonal antibodies against different linear epitopes of E2 protein. E2P3, E2P5, E2P7, E2P9, E2P10, E2P11, E2P13, E2P16, E2P17 peptides showed high antibody levels in outbred as well as H-2d strain and sustained over longer duration. Interestingly all the above peptides showed strong memory response after challenge with native E2 protein which is a prerequisite for any immunogen to act as a potential vaccine.

These results highlight the identification of immunodominant B cell epitopes on E2 protein. In our previous study reactivity of different peptides with human convalescent CHIKV sera showed overlapping sequences with our murine data [24]. Nine peptides
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