Journal of Medical Virology 86:1393–1401 (2014)

Correlation of Plasma Viral Loads and Presence of Chikungunya IgM Antibodies with Cytokine/Chemokine Levels During Acute Chikungunya Virus Infection

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Chikungunya (CHIKV) is an emerging arboviral infection of public health concern in India contributing to widespread morbidity. The precise molecular events occurring early in the infection have not been well understood. Cytokines/chemokines are suspected to play a key role in its pathogenesis. Very few studies have correlated the plasma levels of cytokines/chemokines with diagnostic markers such as viral loads and presence of CHIKV IgM antibodies. Understanding these dynamics in the early phase of CHIKV infection is likely to provide an insight into the evolution of the immune response, identify biomarkers for assessing severity, and for development of newer therapeutic strategies. This study was therefore undertaken to estimate the levels of various cytokines/chemokines in plasma samples of patients infected with CHIKV and correlate to viral load and CHIKV IgM antibodies. Cytokine/chemokine levels and viral loads in plasma were measured using cytometric bead array and TaqMan real time PCR assay, respectively. The findings revealed that acute phase of CHIKV infection is characterized by predominant inflammatory responses mediated by IL-6, IL-8, IP-10, MCP-1, and MIG ($P < 0.003$). Plasma levels of IL-6 ($r = 0.53$, $P < 0.05$) and MCP-1 ($r = 0.83$, $P < 0.05$) emerged as reliable biomarkers of high viral loads in Chikungunya patients. Further, presence of elevated levels of MCP-1 and MIG during the chronic phase of the disease suggests that these chemokines may contribute to perpetuation of symptoms. Hence, these chemokines might serve as targets for the development of treatment to ameliorate the symptoms during the acute phase and prevent the development of chronic manifestations. ***J. Med. Virol. 86:1393–1401, 2014.*** © 2014 Wiley Periodicals, Inc.

KEY WORDS: cytokines/chemokines in Chikungunya; biomarkers of Chikungunya severity

INTRODUCTION

Chikungunya virus (CHIKV) has been the cause of major public health concern in Asia and Africa in the recent years. The virus that belongs to family *Togaviridae* and genus *Alphavirus* has caused massive outbreaks in Reunion islands where 38% of the population was affected and in India where around 1.3 million cases were reported [Ravi, 2006; Borgherini et al., 2007; Pialoux et al., 2007]. The virus has also spread to newer regions like Italy, USA, France, and Japan [Beltrame et al., 2007; Lanciotti et al., 2007; Economopoulou et al., 2009] and the spread has been attributed to increase in global travel and climatic changes. The magnitude of outbreaks and the extent of morbidity caused by CHIKV has drawn attention to this otherwise neglected tropical viral disease. Though the clinical manifestations of Chikungunya have been described in great detail, the exact mechanisms for the pathogenesis of the virus are still poorly understood. Arthralgia, the hallmark of illness due to CHIKV infection has been reported to persist beyond 3 years [Brighton et al., 1983], and indeed there are reports of arthralgia persisting up to 8 years post infection [Brighton, 1984; Waymouth et al., 2013]. Additionally, a recent study suggested

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Accepted 24 November 2013

DOI 10.1002/jmv.23875
Published online 13 February 2014 in Wiley Online Library (wileyonlinelibrary.com).

that CHIKV infection may contribute to the initiation of rheumatoid arthritis [Bouquillard and Combe, 2009] but this finding merits further investigation as there are other studies which have shown that CHIKV induced arthritis can be distinguished from classic rheumatoid arthritis [Manimunda et al., 2010; Ganu and Ganu, 2011]. The host immune mediated mechanisms are suspected to be the cause for the manifestations especially the arthralgia and the arthritic sequelae. Although several studies have reported the cytokine/chemokine profiles during the CHIKV infection [Ng et al., 2009; Hoarau et al., 2010; Chaaitanya et al., 2011; Chow et al., 2011; Wauquier et al., 2011; Lohachanakul et al., 2012], very few of the studies have correlated the levels of cytokines/chemokines to diagnostic markers such as viral loads [Chow et al., 2011], during the acute phase of the CHIKV infection. Correlation of virological and serological markers to the cytokine/chemokine levels early in the course of illness is likely to provide an insight into the evolution of the immune responses. Further, it would also help in elucidating the possible role of cytokines/chemokines serving as potential biomarkers for assessing the disease severity and outcome. Therefore, the present study was undertaken to measure the levels of various cytokines/chemokines in plasma samples of patients with confirmed CHIKV infection. Additionally, the levels of cytokines/chemokines in the acute phase were correlated to the presence or absence of CHIKV RNA and virus specific IgM antibodies in plasma.

MATERIALS AND METHODS

Patients and Samples

The study was carried out using samples received at the Department of Neurovirology, National Institute of Mental Health and Neurosciences (NIMHANS). The study was approved by institute ethics committee (NIMHANS/68th IEC/2010). After obtaining informed consent, 48 subjects were enrolled into the study. Suspected patients infected with CHIKV presented to the hospital or clinics attached to the laboratory with fever, joint pain, rash, myalgia, conjunctival redness, and headache. Additionally, the prevalence of local outbreaks in the region aided in making a clinical diagnosis of Chikungunya fever. Blood samples (3–5 ml of blood) were collected from the 48 subjects, plasma separated and stored in aliquots at −70°C until all tests were performed. All the samples were collected between 2 and 10 days after the onset of symptoms. The CHIKV infection was confirmed by detection of CHIKV specific IgM antibodies using an ELISA (National Institute Virology, Pune) and/or CHIKV RNA by TaqMan real time PCR targeting the NSP4 region as described earlier [Reddy et al., 2012]. Further, the CHIKV load in the plasma samples was also quantified using TaqMan real time PCR as described earlier [Reddy et al., 2012]. Plasma samples collected from 37 healthy individuals served as controls. They were recruited specifically for the study after appropriate screening and were age and sex matched. The pre- requisites for inclusion in the study was no history of febrile illness for the past 3 weeks at the time of inclusion and a negative result for CHIKV IgM antibodies using ELISA.

Chikungunya confirmed patients were divided into two groups; Group 1—Patients who were positive for CHIKV RNA (viraemic); Group 2—Patients who were negative for CHIKV RNA but positive for CHIKV specific IgM antibody (non- viraemic).

Amongst the 48 patients included in the study, serial samples could also be obtained from four patients who developed arthralgia that was persistent beyond 2 months post onset of symptoms. Samples were collected from these patients within the first week after onset of symptoms and subsequently during the second week, 4 weeks and 12 weeks after onset of symptoms.

Taqman Real Time PCR

The TaqMan real time PCR was carried out using CHIKV RNA specific primers and probes in the genomic region coding for the non-structural protein 4 (NSP4) as described earlier [Reddy et al., 2012]. Briefly, RNA was extracted from 140 μl of plasma samples using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany). RNA was converted to cDNA using High capacity cDNA reverse transcription kit (Applied Biosystems, Fostercity, CA) according to manufacturer's instructions and was evaluated in TaqMan real time PCR assay. Cycle threshold (Ct) values obtained were plotted against the log dilutions of the virus for the construction of the standard curve. The copy number in the plasma samples was calculated by extrapolating the Ct values obtained on the standard curve.

ELISA for IgM Antibody Detection

The samples were tested for the presence of CHIKV specific IgM antibodies using the IgM Capture ELISA kit from National institute of Virology (NIV), Pune, India [Reddy et al., 2012]. Briefly, 50 μl of plasma sample (1:100 dilution) along with positive and negative controls provided in the kit was added to anti human IgM coated microwells and incubated for 1 hr. This was followed by the addition of 50 μl of CHIKV antigen and incubated for 1 hr. Unbound antigen was removed by washing and 50 μl biotinylated anti-CHIKV antibody was added, incubated for 1 hr. The wells were washed and 50 μl of avidin- HRP was added and incubated for 30 min. Subsequently 100 μl of substrate was added and incubated for 10 min in the dark for color development. The reaction was stopped by the addition of 100 μl of 1N sulphuric acid and absorbance was measured at 450 nm. The results were interpreted according to the manufacturer's instructions provided in the kit.

Estimation of Cytokine/Chemokine Levels in Plasma Samples

Commercial cytokine Bead Array (CBA) based kits (BD Biosciences, San Jose, CA) were used for estimation of plasma levels of eleven cytokines (IL-1ß, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-17, IFN-γ, and TNF-α) and four chemokines (IP-10, MIG, MCP-1, and RANTES). Briefly, 50 μl of a mixture of bead population with distinct fluorescence intensities, coated with specific antibodies for capturing different cytokines was incubated with 50 μl of plasma for 1.5 hr at room temperature in the dark. The complexes were washed with the wash buffer provided in the kit and the cytokine-captured beads were mixed with 50 μl of phycoerythrin-conjugated detection antibodies to form sandwich complexes. After incubation for 1.5 hr at room temperature in the dark, followed by a washing step, the samples were subjected to flow cytometry (FACSCalibur system, BD biosciences, San Jose, CA) and the data was acquired for the samples. In each run of the assay cytokine/chemokine standards provided by the manufacturer were included to construct standard curves using the BD CBA software. The data from the standard curve was used to extrapolate the level of cytokines/chemokines in the clinical samples and the levels were expressed as picogram per milliliter (pg/ml).

Statistical Analysis

Comparison of plasma cytokine/chemokine levels between CHIKV patients and healthy controls was carried out using the Mann–Whitney test with Bonferroni's corrections. The level of cytokines/chemokines was also compared among the patients infected with CHIKV (viraemic versus non-viraemic) as well as those with high viral load and low viral load using Mann–Whitney test with Bonferroni's correction. P values <0.003 were considered significant. The cytokines/chemokines that were significantly different between the high viral load and low viral loads subgroups were correlated to plasma viral loads using the Pearson's correlation analysis. P value of <0.05 was considered significant. The levels of correlation were interpreted as described earlier by Zou et al. [2003]. Interpretation was based on correlation coefficient values: (i) 0 = no correlation, (ii) 0.2 = weakly positive, (iii) 0.5 = moderately positive, (iv) 0.8 = strong positive, and (v) 1.00 = perfectly positive correlation.

RESULTS

Socio Demographic and Clinical Data

All the patients and the controls in the study were from the state of Karnataka, South India. Among the 48 patients with confirmed CHIKV infection, 17 were females and 31 were males. The mean age of the all patients infected with CHIKV was 40.6 years (range = 21–80), while it was 52.5 years (range = 42–57) for the patients who developed persistent arthralgia. The mean age of the subjects in the control group was 39.7 years and included 23 males and 14 females. Blood samples were collected from the all the subjects between Day 2 and 10 post onset of symptoms. All the patients presented with fever, while 40 (83%) had arthralgia, 30 (63%) had myalgia, and 20 (42%) complained of headache. Rash and gastrointestinal symptoms were seen in 14 (29%) of the patients each, and conjunctival redness was seen in 3 (6%) of the patients (Table I).

Virological and Serological Findings

Amongst the 48 patients included in the study, 29 patients who were positive for CHIKV RNA were placed in Group 1, 19 patients negative for CHIKV RNA but positive for CHIKV specific IgM antibodies were placed in Group 2. Further, Group 1 (n = 29) was further divided into high viral load (high viral load, $>10^5$ copies/ml, n = 19) and low viral load (low viral load, $<10^5$ copies/ml, n = 10) subgroups. The classification of the patients into high viral load and low viral load subgroups in the study are related to time of sampling rather than the severity of the infection in terms of viral loads. Mean viral load in high viral load of CHIKV RNA positive patients was 3.85×10^6 copies/ml (range: 9.4×10^6–1.2×10^5 copies/ml), while it was 2.18×10^4 copies/ml (range: 8.4×10^4–3.1×10^3 copies/ml) in the low viral load subgroup. Further, 4/19 (21%) patients with high viral load and 9/10 (90%) patients with low viral load were positive for CHIKV specific IgM antibodies (Fig. 1).

Comparison of Cytokine/Chemokine Levels Between the Chikungunya Confirmed Patients and Healthy Controls

The mean levels of cytokines/chemokines determined in the plasma samples of the Chikungunya confirmed patient group and healthy controls is summarized in the Table II. Of the eleven cytokines studied, the levels of four cytokines—IL-4, IL-5, IL-6, and IL-8 were significantly elevated ($P < 0.003$) in the Chikungunya confirmed patient group (n = 48) as compared to the healthy control group. Among these, the levels of IL-6 and IL-4 (mean = 382.5 ± 1215.4 and 331.4 ± 1218.7 pg/ml, respectively) were several

TABLE I. Summary of Salient Clinical Features in Patients with Laboratory Confirmed CHIKV Infection

Symptoms	Number of cases (%)
Fever	48 (100)
Rash	14 (29)
Myalgia	30 (63)
Arthralgia	40 (83)
Conjunctival redness	3 (6)
Gastrointestinal symptoms	14 (29)
Headache	20 (42)

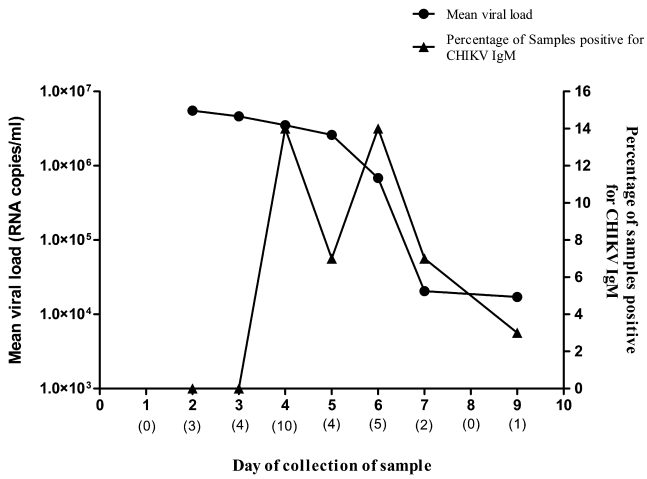

Fig. 1. Graph depicting the kinetics of viral load and CHIKV IgM antibodies in relation to duration of illness (n = 29). X-axis depicts the day of collection of sample. The Y-axis on the left depicts the mean viral load (RNA copies/ml), while the Y-axis on the right depicts the percentage of samples positive for CHIK IgM antibodies. The numbers in the parenthesis indicated below the X-axis represents the number of samples tested on respective day.

fold high (>100) and the levels of IL-8 (mean = 598.0 ± 1537.7 pg/ml) were moderately elevated (>50) in the patient group. Further, the levels of IL-5 (mean = 52.7 ± 287.8 pg/ml) were 22-fold higher in the patient group.

Among the four chemokines studied, the levels of MCP-1, MIG and IP-10 were found to be significantly elevated in the patient group ($P < 0.003$) as compared to healthy control group. The mean level of MCP-1, MIG, and IP-10 was 424.0 ± 388.7, 631.1 ± 423.1, and 746.8 ± 463.7 pg/ml respectively, while the mean level of these chemokines in the control group was found to be 38.7 ± 13.7, 51.6 ± 107.0, and 161.0 ± 68.9 pg/ml respectively. However, the level of the chemokine RANTES in the plasma samples from the Chikungunya confirmed patient group (mean = 629.3 ± 375.7 pg/ml) was significantly lower ($P < 0.003$) compared to the healthy group (mean = 1,250 ± 0 pg/ml).

Correlation of Cytokines/Chemokines to Plasma CHIKV Viral Loads

Cytokines. The comparison of the plasma proinflammatory cytokine levels of IL-6 and IL-8 between Chikungunya patients positive for viral RNA and those negative for viral RNA were significantly different (Table III). None of the Th1 and Th2 cytokines were found to be significantly different between the two patient groups. Analysis of cytokine levels within the CHIKV RNA positive group of patients revealed that IL-6 and IL-8 levels were significantly higher ($P = 0.002$ for IL-6 and $P = 0.001$ for IL-8) in patients with high viral load as compared to those with low viral load (Fig. 2). Further, Pearson correlation analysis revealed that levels of IL-6 showed a moderate correlation ($r = 0.53$, $P = 0.020$) with viral load (Panel A, Fig. 3). The same was not noted with IL-8 ($r = 0.28$, $P > 0.05$).

Chemokines. The levels of all the four chemokines IP-10, MIG, MCP-1, and RANTES were found to be significantly higher ($P < 0.003$) in patients

TABLE II. Comparison of Cytokine/Chemokine Levels in Patients Infected with CHIKV and Healthy Controls

	CHIKV infected patient group		CONTROL[a]			
Cytokine/ chemokine	Mean ± SD Range in parentheses (range in pg/ml)	Median	Mean ± SD Range in parentheses (range in pg/ml)	Median	*P* value	Mean fold ratio[b]
IL-1β	127.7 ± 730.3 (0–5,000)	2.0	2.3 ± 2.2 (0–6.3)	2.7	0.675	55.5
IL-2	2.2 ± 0.8 (0–3.6)	2.2	2.2 ± 1.0 (0–4.2)	2.3	0.729	1.0
IL-4	331.4 ± 1218.7 (0–5000)	8.3	3.0 ± 1.1 (1.4–6.6)	2.7	**0.001**	110.4
IL-5	52.7 ± 287.8 (0–1998.5)	3.4	2.4 ± 0.9 (0–4.7)	2.4	**0.001**	22.0
IL-6	382.5 ± 1215.4 (0–5000)	8.8	2.1 ± 1.7 (0–6.4)	2.6	**0.001**	182.1
IL-8	598.0 ± 1537.7 (1.7–5,000)	14.2	8.8 ± 14.3 (0–75.9)	4.9	**0.001**	68
IL-10	56.7 ± 324.7 (0–2253.5)	2.5	1.7 ± 1.6 (0–5.5)	2.2	0.007	33.4
IL-12p70	1.6 ± 1.4 (0–4.9)	1.7	2.2 ± 2.1 (0–5.6)	2.5	0.149	0.7
IL-17	1.9 ± 0.9 (0–3.8)	1.9	1.8 ± 1.1 (0–4.1)	2.1	0.569	1.0
IFN-γ	10.0 ± 9.5 (0–47.9)	9.0	10.5 ± 10.4 (0–32.5)	8.5	0.968	1.0
TNF-α	9.7 ± 41.9 (0–275.8)	1.6	1.7 ± 1.6 (0–4.6)	2.4	0.909	5.7
IP-10	746.8 ± 463.7 (36.1–1,250)	666.7	161.0 ± 68.9 (6.6–289.9)	137.3	**0.001**	4.6
MIG	631.1 ± 423.1 (46.7–1450.3)	486.0	51.6 ± 107.0 (3.5–669.0)	31.5	**0.001**	12.2
MCP-1	424.0 ± 388.7 (28.0–1,250)	261.1	38.7 ± 13.8 (8.2–66.9)	34.9	**0.001**	11
RANTES[d]	629.3 ± 375.7 (77.9–1,250)	507.1	≥1,250[c] (1,250–1,250)	1250.0	**0.001**	0.5

[a]Controls comprised of age and sex matched healthy adults (n = 37) for details see Materials and Methods Section; *P* values of statistical significance are shown in bold font.
[b]Mean values of patients/Mean values of controls.
[c]The samples were not diluted further and tested.
[d]The platelet of CHIKV patients in the study ranged from 112,000 to 410,000 (Mean = 1,82,905/mm^3) and they were closer to the lower limit of the acceptable normal range for Indian subjects (150,000–250,000/mm^3). The platelets of all the control were within the normal limits and ranged from 180,000 to 250,000/mm^3.

TABLE III. Cytokine/Chemokine Levels in Two Groups of Chikungunya Patients

Cytokine/ chemokine	Group1 (n = 29) (Positive for CHIKV RNA) Mean ± SD range in parentheses (range in pg/ml)	Median	Group 2 (n = 19) (Negative for CHIKV RNA but positive for CHIKV IgM antibodies) Mean ± SD range in parentheses (range in pg/ml)	Median	*P* value
IL-1β	209.6 ± 936.8 (0.0–5,000)	2.0	2.6 ± 5.0 (0.0–22.95)	1.7	0.173
IL-2	2.5 ± 0.8 (0.0–3.50)	2.5	1.8 ± 0.8 (0.0–3.7)	1.9	0.005
IL-4	361.1 ± 1285.2 (2.2–5000)	8.9	286.1 ± 1142.4 (0.0–5000)	6.4	0.759
IL-5	80.6 ± 369.9 (1.6–1999.0)	3.6	10.1 ± 16.4 (0.0–68.0)	3.3	0.650
IL-6	677.9 ± 1261.3 (0.0 ± 5,000)	164.9	12.8 ± 17.1 (0.0–65.8)	6.2	**0.002**
IL-8	734.1 ± 1721.9 (1.7–5,000)	27.4	121.8 ± 491.6 (2.8–2151.6)	6.2	**0.001**
IL-10	90.90 ± 417.0 (0.0–2253.5)	3.0	4.5 ± 6.7 (0.0–27.6)	2.3	0.116
IL-12p70	1.60 ± 1.3 (0.0–4.9)	1.8	1.60 ± 1.5 (0.0–4.8)	1.6	0.846
IL-17	2.0 ± 0.8 (0.0–2.9)	2.1	1.7 ± 0.9 (0.00–3.8)	1.6	0.064
IFN-γ	12.6 ± 10.8 (0.0–47.9)	10.4	6.2 ± 5.5 (0.0–17.1)	6.2	0.020
TNF-α	15.1–107.1 (0.0–275.8)	1.7	1.5 ± 2.00 (0.0–6.8)	1.3	0.131
IP-10	291.8 ± 460.0 (3.4–1,250)	72.8	88.4 ± 281.7 (7.8 ± 1,250)	18.5	**0.002**
MIG	791.7 ± 434.9 (46.7–1,250)	770.0	385.9 ± 261.3 (140.6–1,250)	295.7	**0.001**
MCP-1	545.4 ± 418.9 (28.0–1,250)	412.7	238.9 ± 259.0 (78.2–1,250)	160.2	**0.002**
RANTES	766.6 ± 386.4 (77.9–1,250)	734.3	419.7 ± 244.2 (189.4–1,250)	341.6	**0.002**

P values of statistical significance are shown in bold font.

positive for CHIKV RNA as compared to those patients negative for CHIKV RNA (Table III). Further analysis of chemokine levels within the CHIKV RNA positive group of patients (Fig. 2) revealed that levels of MCP-1, MIG, and RANTES were significantly higher in high viral load as compared to low viral load subgroup ($P = 0.002$ for MCP-1; $P = 0.002$ for MIG and $P = 0.002$ for RANTES). In the Pearson correlation analysis a very strong relationship emerged between plasma viral load and MCP-1 levels ($r = 0.83$, $P < 0.05$) in the viraemic group. Further this correlation was explored in the high viral load and the low viral load subgroups individually. The correlation between plasma viral loads and MCP-1 levels appeared stronger ($r = 0.93$, $P < 0.0001$) in the HIGH VIRAL LOAD subgroup (Panel B, Fig. 3) as compared to that in the LOW VIRAL LOAD subgroup ($r = 0.05$).

Cytokine/Chemokine Levels During the Phase of Persistent Arthralgia

The mean age of the four patients who presented with persistent arthralgia was 52.5 years (range = 42–57). In the four patients who presented with persistent arthralgia, it was noted that the median values of IL-1β, IL-4, IL-6, IL-8, IP-10, MCP-1, MIG, and RANTES and were highest during the second week of illness. Thereafter the levels of these cytokines/chemokines returned to near normal values except MIG, MCP-1, and RANTES (Table IV). Amongst these three chemokines, the median levels of MIG was 720 pg/ml within the first week of illness, 739 pg/ml during the second week, and thereafter declined to 433 pg/ml at 8 weeks and 238 pg/ml at 12 weeks after onset of symptoms. Plasma levels of MCP-1 also exhibited a similar pattern with median levels of 386 pg/ml during the first week, 687 pg/ml during the second week, and thereafter declining to 233 pg/ml at 8 weeks and 129 pg/ml at 12 weeks. It was noteworthy that throughout the follow-up period of these four subjects MIG and MCP-1 levels remained elevated as compared to control subjects (Table IV). On the other hand, the median levels of chemokine RANTES showed a marked declining pattern from the first week of illness until the end of follow up period (12 weeks).

DISCUSSION

CHIKV is one of the emerging arboviral infections of public health importance in India following the massive outbreaks it caused in the subcontinent from 2006 [Ravi, 2006]. Several studies have been carried out to investigate the precise cellular and molecular basis of persistent arthralgia in CHIKV infection. These include the presence of rheumatoid factor (RF), HLA DRB1*04 or 01 alleles [Bouquillard and Combe, 2009], HLA B27 [Chopra et al., 2012], cyroglobulins [Oliver et al., 2009], and cytokine/chemokine levels [Dupuis-Maguiraga et al., 2012]. Amongst these, the estimation of acute inflammatory mediators during the various stages of CHIKV infection has received widespread attention from various investigators [Ng et al., 2009; Chaaitanya et al., 2011; Chow et al., 2011; Wauquier et al., 2011]. While there has been some degree of concordance between the plasma cytokines/chemokine levels (IL-6, IL-8, IP-10, MIG, and MCP-1) noted in acute phase of CHIKV infection in several studies [Chaaitanya et al., 2011; Chow et al., 2011; Wauquier et al., 2011], there have been conflicting observations with respect to the plasma levels of IFN-γ and RANTES [Wauquier et al., 2011]. In addition, despite the widespread morbidity

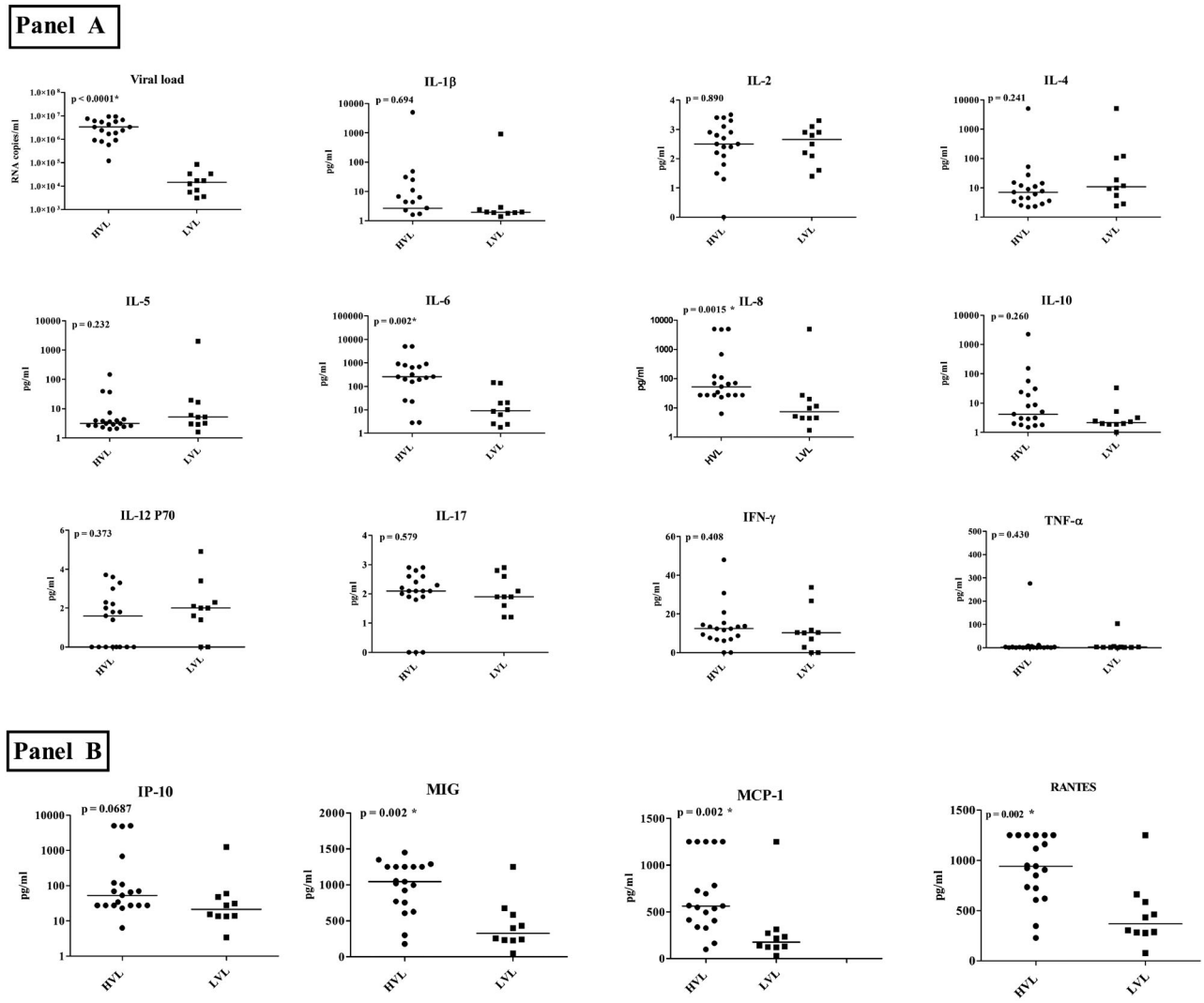

Fig. 2. Scatter plots depicting the levels of cytokines (**Panel A**) and chemokines (**Panel B**) amongst the two subgroups of CHIKV RNA positive patients. X-axis represents the two subgroups HVL—high viral load and LVL—low viral load. Y—axis represents the levels of cytokines/chemokines in pg/ml. *P* values <0.003 were considered significant (*). The horizontal bar represents the median values in each subgroup.

induced by Chikungunya in India, there has been only a single study [Chaaitanya et al., 2011] that has investigated the role of cytokines/chemokines in CHIKV infection. Therefore, the present study was undertaken to determine the levels of various cytokines/chemokines in patients with CHIKV infection during the acute phase of illness (2–10 days post onset of illness).

This study revealed that the levels of 4/11 cytokines (IL-4, IL-5, IL-6, and IL-8) were found to be significantly elevated in Chikungunya patients as compared to controls subjects ($P < 0.003$). Further, significantly higher levels of IL-6 and IL-8 were noted in patients positive for CHIKV RNA as compared to those negative for CHIKV RNA (Table III). The levels of these two cytokines were also significantly elevated in the high viral load subgroup as compared to the low-level viral load subgroup (Fig. 2). Elevated levels of IL-6 during acute phase of CHIKV infection in comparison to healthy controls has been reported in several earlier studies [Ng et al., 2009; Chaaitanya et al., 2011; Chow et al., 2011; Kelvin et al., 2011; Lohachanakul et al., 2012]. Amongst these, only one study carried out in patients from Singapore [Chow et al., 2011] was similar in design to the present study as it investigated cytokine/chemokine levels early in the acute phase of Chikungunya illness. Indeed, Chow et al. [2011] had also observed a significant correlation of IL-6 levels with a high viral load. Similar correlation between CHIKV and IL-6 was also reported in Macaque models for CHIKV during peak viremia [Chen et al., 2010; Labadie et al., 2010; Messaoudi et al., 2013]. Similarly in this study, a positive correlation albeit moderate, was

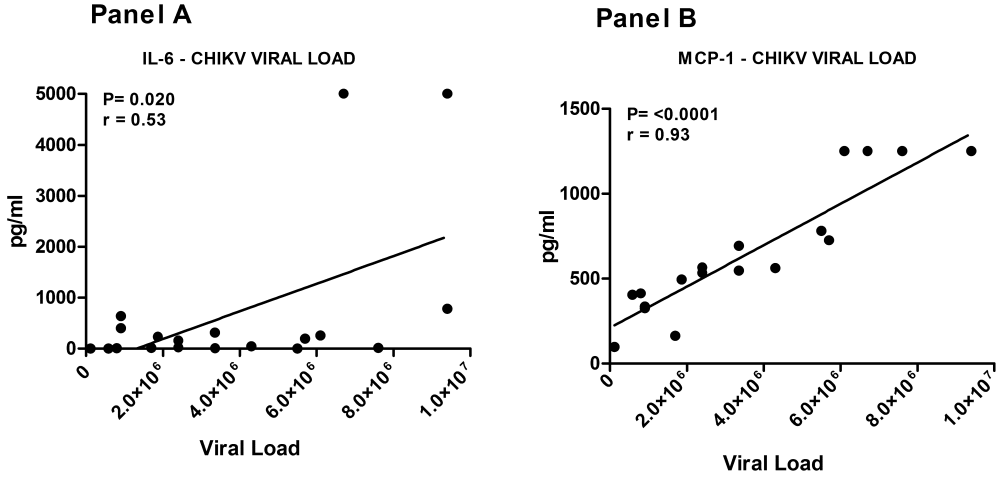

Fig. 3. Scatter plot depicting the correlation between IL-6 and CHIKV viral load (**Panel A**) and MCP-1 and CHIKV viral load (**Panel B**). X-axis represents the CHIKV viral load, while Y-axis represents the level of cytokine in pg/ml. Pearsons correlation coefficient and *P* value is given in the respective graph. Each point corresponds to one patient. The solid line shows the best line fitted to the data based on simple regression model.

noticed between IL-6 levels and viral load. Thus it appears that IL-6 is a reliable surrogate marker of viral load in the early phase of Chikungunya illness. Plasma levels of IL-8 in this study were significantly higher in the high viral load subgroup of patients ($P = 0.0015$). Chow et al. [2011] on the contrary, did not find statistically significant levels of IL-8 in patients with high viral load. The level of IL-4 in patients infected with CHIKV was more than 100-fold higher and that of IL-5 was around 20-fold higher as compared to healthy controls ($P < 0.05$). These Th2 cytokines contribute to the development and maturation of B-cells and thereby play an important role in antibody mediated immune response which might contribute to the decrease in the viremia during the course of illness and high levels of IgG at a later stage of the disease [Hoarau et al., 2010].

TABLE IV. Cytokine/Chemokine Levels in Patients with Persistent Arthralgia Followed Up for 12 weeks (n = 4)

Median values for various cytokines/chemokines (pg/ml)					
		Time of collection of serial sample			
Cytokine/ chemokine	Controls	1 week	2 weeks	4 weeks	12 weeks
IL-1β	2.7	2.4	12.4	2.4	2.0
IL-2	2.3	2.6	2.7	2.2	1.7
IL-4	2.7	3.5	18.3	2.8	18.5
IL-5	2.4	3.5	5.0	2.1	2.7
IL-6	2.6	14.8	34.1	2.5	2.6
IL-8	4.9	31.1	1078.0	11.5	4.6
IL-10	2.2	2.4	1.8	2.4	1.8
IL-12p70	2.5	1.4	1.0	1.9	2.0
IL-17	2.1	2.0	2.7	1.8	1.2
IFN- γ	8.5	12.4	7.8	9.8	2.8
TNF-α	2.4	1.4	4.3	1.4	1.5
IP-10	137.3	65.8	631.7	31.2	14.1
MIG[a]	**31.5**	**720.3**	**739.1**	**433.3**	**238.4**
MCP-1[a]	**34.9**	**386.1**	**687.3**	**233.1**	**129.9**
RANTES[b]	**1250**	**695.8**	**764.1**	**462.9**	**288.0**

No statistical test of significance was applied to the data due to small sample size. The data presented is only descriptive.
Bold font indicates the chemokines whose levels did not return to near normal limits at the end of 12 weeks.
[a]The median values of MCP-1 and MIG remained higher in these patients when compared to the controls throughout the follow up period of 12 weeks.
[b]The median values of RANTES in these patients was lower compared to the controls throughout the follow up period of 12 weeks.

The levels of other cytokines such as IL-2, IL-10, IL-12, IL-17, and TNF-α were not significantly different between Chikungunya patients and healthy controls. This observation is in agreement with two earlier studies [Ng et al., 2009; Chow et al., 2011] but in variance with that reported by Hoarau et al. [2010] and Wauquier et al. [2011] who observed that plasma levels of IFN-γ were significantly higher in CHIKV patients (during the acute phase of illness) as compared to controls.

The levels of MCP-1, MIG, and IP-10 were also significantly elevated in the CHIKV infected patient group ($P < 0.05$) as compared to controls. In addition, the level of MCP-1 was significantly higher in the patients positive for CHIKV RNA (Table III). The high viral load subgroup of patients also exhibited a significantly higher ($P = 0.003$) level of this chemokine as compared to patients in the low viral load subgroup. High plasma levels of MCP-1 has been reported during the acute phase of CHIKV infection in earlier studies [Chaaitanya et al., 2011; Wauquier et al., 2011] and a strong correlation observed with high viral loads [Chow et al., 2011]. Amongst all the cytokines/chemokines measured in this study, MCP-1 was the only chemokine that exhibited a very strong correlation to plasma viral load ($r = 0.83$). The

relationship between MCP-1 levels and viral load was stronger in the high viral load subgroup ($r = 0.93$, $P < 0.0001$) as compared to low viral load subgroup ($r = 0.05$). A similar finding was reported in Macaque model for CHIKV where peak viremia was characterised by high levels of MCP-1 [Chen et al., 2010; Labadie et al., 2010; Messaoudi et al., 2013].

High levels of MCP-1 were noted in the four patients who had persistent arthralgia lasting for 3 months following CHIKV infection (Table IV). MCP-1 is involved in recruitment and activation of macrophages and has also been implicated in pathogenesis of other arthritogenic alpha viral infections such as Ross River Virus [Lidbury et al., 2008]. In addition, it has been reported that the elevated plasma MCP-1 levels in preclinical asymptomatic seropositive rheumatoid arthritis subjects serves as reliable biomarker predicting progression to disease state [Rantapää-Dahlqvist et al., 2007; Kokkonen et al., 2010]. Therefore, MCP-1 might serve as a reliable biomarker of CHIKV induced persistent arthritis. However, in the absence of comparison with the MCP-1 levels in the follow up samples from patients who recovered from Chikungunya illness, no direct significance can be attributed to MCP-1 and persistent arthralgia. In an earlier study interestingly, inhibition of MCP-1 by anti-inflammatory drug, Bindarit, led to the amelioration of inflammation in joint and skeletal muscles in mouse model of CHIKV [Rulli et al., 2011].

Significantly elevated MIG levels were noted in Chikungunya patients as compared to controls, as well as in CHIKV RNA positive patients as compared to CHIKV RNA negative patients. Although the levels of this chemokine were significantly different between high viral load and low viral load subgroups (Fig. 2) it did not exhibit any correlation to viral loads ($r = 0.17$). Kelvin et al. [2011] also reported high levels of MIG in patients during the acute phase. Further, they proposed that this chemokine can serve as a potential biomarker for assessing severity of CHIKV infection as it contributes to persistent immune activation leading to chronic symptoms. These observations are in part supported by the findings of the present study wherein MIG levels continued to be elevated in the four patients who had persistent arthralgia and were followed up for 12 weeks (Table IV).

Amongst all the cytokines/chemokines estimated in the present study, RANTES was the lone immune marker which was consistently lower in Chikungunya patients during the acute phase (Table II) as well as in the four patients who had persistent arthralgia (Table IV). However, there was no significant correlation ($r = 0.22$, $P > 0.05$) observed between RANTES and CHIKV viral load. The only other study which reported a similar observation of decreased plasma RANTES level in patients infected with CHIKV was also from India [Chaaitanya et al., 2011]. Furthermore, the patients investigated by them were also from the same Southern State of India where the present study was conducted. All the other studies carried out elsewhere did not find any significant difference in plasma RANTES levels between patients infected with CHIKV and healthy controls. Therefore, it is tempting to postulate that this difference may reflect regional characteristics of the cohorts studied, with different genetic backgrounds. Alternatively, the low levels of RANTES noted in the Indian patients reflect severity of CHIKV infection. RANTES levels are lower in CHIKV infected subjects who have thrombocytopenia as platelets are the storehouse of this chemokine [Ng et al., 2009]. It was interesting to note that RANTES levels continued to remain low even at 12 weeks after onset of symptoms in the four subjects who presented with persistent arthralgia. It would therefore be imperative to follow a large cohort of patients infected with CHIKV to determine if this chemokine would emerge as a biomarker of severity and chronicity in the disease process.

In conclusion, the study has attempted to give an insight into the nature of immune responses mediated through cytokines/chemokines during the acute phase of CHIKV infection (sampling time 2–10 days). The findings in the study reflect prominent inflammatory responses in the very early stage of the disease as evidenced by high levels of IL-6, IL-8, IP-10, MCP-1, and MIG. During analysis of the levels of cytokines/chemokines, IL-6 and MCP-1 emerged as markers of high viral load. Amongst these two, MCP-1 showed a very strong correlation to high viral load and might serve as a reliable marker of high viral load in Chikungunya patients. Further, in four patients with chronic arthralgia the levels of chemokines MCP-1 and MIG remained elevated suggesting an important role for them in perpetuation of symptoms. Hence, these chemokines might serve as important targets for development of therapeutics, which contribute to ameliorate the symptoms during the acute phase and prevent the development of chronic manifestations.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Subbakrishna and Dr. Mariamma Philip from the Department of Biostatistics, National Institute of Mental Health and Neurosciences (NIMHANS) for the assistance with the statistical analysis in this study.

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