Inflammatory Cytokine Expression Is Associated with Chikungunya Virus Resolution and Symptom Severity

Alyson A. Kelvin1,2, David Banner3, Giuliano Silvi4, Maria Luisa Moro5, Nadir Spataro6, Paolo Gaibani6, Francesca Cavrini6, Anna Pierro6, Giada Rossini6, Mark J. Cameron3, Jesus F. Bermejo-Martin7,8, Stéphane G. Paquette3,9, Luolong Xu3, Ali Danesh3, Amber Faroqui7, Ilaria Borghetto1,10, David J. Kelvin3,9,11, Vittorio Sambri6, Salvatore Rubino1,10

Introduction

The Chikungunya virus (CHIKV), an arthropod-borne virus (arbovirus), is a single-stranded positive-sense RNA virus with three genotypes. The virus is of the Alphavirus genus in the Togaviridae family [1,2]. CHIKV has been shown to infect and be transmitted by Ae. aegypti and Ae. albopictus mosquitoes. It was identified in East Africa in the early 1950s and since then has caused epidemics in continental Africa, the Indian Ocean region, and countries of Southeast Asia such as India, where since 2006 suspected cases have been estimated to be 1.39 million, and Singapore [3–6]. The only reported outbreak outside these areas was in Italy in the Emilia Romagna region in 2007. Small non-epidemic imported cases have been reported in other regions such as North America, France and Japan, which were caused by travelers returning from affected areas [7–9].

The epidemic occurring on La Reunion Island in the Indian Ocean remains the most devastating of all CHIKV outbreaks where over one-third of the population was affected [10]. During this outbreak, the CHIKV acquired a genetic mutation allowing the new vector Ae. albopictus mosquito to carry the virus where previously CHIKV only circulated in Ae. aegypti mosquitoes [10,11]. The Ae. albopictus differs in susceptibility to various genetically different isolates of the virus compared to the Ae. aegypti [12]. CHIKV is now of global health concern since expansion of mosquito vectors has created potential for the Chikungunya virus to spread to temperate areas as Ae. albopictus inhabits regions in North America and Europe [2,13].

CHIKV infection is clinically characterized by the sudden appearance of high fever, rash, headache, nausea, vomiting, myalgia and arthralgia or severe joint pain. Severe joint pain is the defining symptom of CHIKV disease [11]. The word Chikungunya originated from the Tanzanian and Mozambique region of Africa meaning that which bends. A bent posture is often taken by those previously affected in CHIKV infection.
Author Summary

Chikungunya virus (CHIKV) is transmitted by mosquitoes and causes a human disease clinically characterized by sudden appearance of high fever, rash, headache, nausea, and severe joint pain (the defining symptom). Chikungunya was identified in Africa and the word Chikungunya means that which bends up, describing the bent posture of CHIKV patients while in severe pain. CHIKV, a current problem in Africa, Indian Ocean region, and Southeast Asia, is now spreading to temperate regions of North America, France and Italy. Presently, the immune response for CHIKV infection remains largely uninvestigated and no treatment is available. We investigated cytokine profiles at diagnosis and follow-up of CHIKV infected patients during the Italian 2007 outbreak and associated cytokine levels with antibody level and symptom severity. Cytokines, important immune mediators, are often drug targets. Since CHIKV symptoms can persist for months or years following infection it is important to investigate possible drug targets to alleviate discomfort. We found cytokine profiles that describe the initial infection and recovery phase. We determined the cytokines CXCL9/MIG and CXCL10/IP-10 as well as antibody levels were associated with symptom severity. These results reflect previously unreported cytokine profiles which may be important for the development of future therapeutics for CHIKV outbreaks.

Inflammatory Cytokines Resolve Chikungunya Disease

Materials and Methods

Ethics statement

Patients all gave written consent to the participation in scientific studies. Permission to perform scientific studies was given by Comitato Etico di Area Vasta Romagna Et IRST of the Servizio Sanitario Regionale Emilia-Romagna, Italy.

Objectives

Since the immune response during CHIKV disease has not been extensively investigated, our objectives were to create a clear clinical picture of CHIKV disease at the acute phase and during convalescence at 6- and 12-month follow-up by cytokine profiling. To achieve this objective, we investigated the cytokine profiles from patients at the acute phase and at 6- and 12-month follow-up.

Participants

Included patients were from the region of Emilia-Romagna in north-east Italy suspected to be infected with CHIKV since they showed symptoms such as myalgia, severe back and joint pain, headache, and skin rash. Collaboration with the regional microbiology reference laboratory of Bologna University and the Department of Infectious and Parasitic Diseases of the National Institute of Health in Rome was initiated and identified the patients as having CHIKV. The clinical criteria was described as acute onset of fever (>38.5°C) and severe arthralgia not explained by other medical conditions. CHIKV infection was confirmed by one or more of the acute phase tests: virus isolation, reverse transcriptase-PCR (RT-PCR) positive for CHIKV npl gene, seroconversion to virus-specific serum antibodies collected at least 1 to 3 weeks apart, or presence of virus-specific IgM antibodies in a single serum sample collected [15]. Acute CHIKV patient samples were determined to be in the viral stage if the sample was PCR positive for CHIKV (7 patients), in the IgM antibody initiation stage if the sample was PCR negative, IgM positive, IgG negative (6 patients) or in the seroconversion stage (22 patients) if the sample was PCR negative, IgM positive and IgG positive. The samples were considered to be high IgG if the IgG level was greater than 6400 (6 months) (31 patients high out of 50) or greater than 3200 (12 months) (20 patients out of 50). IgG levels below or equal to these thresholds were considered low IgG. CHIKV patients were considered to be non-symptomatic (15 patients out of 50) at the 6-month follow-up; 34 patients out of 50 at the 12 month follow-up), have mild symptoms (21 patients out of 50 a the 6 month follow-up; 14 patients out of 50 at the 12 month follow-up), have severe symptoms (14 patients out of 50 at the 6 month follow-up; 2 patients out of 50 at the 12 month follow-up) or have severe symptoms (14 patients out of 50 at the 6 month follow-up; 2 patients out of 50 at the 12 month follow-up) based on their responses to a questionnaire at the time of sampling which was based on: arthralgic pain, muscle pain, mono-arthritis, oligo-arthritis, symmetric polyarthritis, asymmetric polyarthritis, tenosynovitis, arthralgia and fibromyalgia. Control samples were collected from 10 healthy volunteers screened for symptoms of viral infection.

Patient sample collection

Blood samples were collected from consenting CHIKV positive patients at the time of diagnosis. Viral infection was determined as described above. Two follow-up samples were then collected from each patient at the 6-month evaluation and the 12-month
Inflammatory Cytokines Resolve Chikungunya Disease

CBA

Serum samples were analyzed for cytokine levels using BD™ Cytometric Bead Array (CBA) Human Chemokine Kit, Human Inflammatory Cytokine Kit, and Human Th1/Th2 Cytokine Kit (BD Biosciences) according to the manufacturer's instructions for a total of 13 cytokines. Capture Beads were added to the serum sample followed by the PE detection reagent. The samples were then incubated for 3 hours at room temperature and washed with the assay Wash Buffer and resuspended again in Wash Buffer for analysis on the Flow Cytometer. CBA data was then run on a BD FACSCalibur Flow Cytometer.

Statistical analysis

CBA data was analysed using SPSS statistical software. Box-whisker plots were created from the CBA FACS raw data. Six-month and 12-month samples were compared to the acute samples using the non-parametric two-tailed Wilcoxon signed-rank test for related samples to determine statistical significance. Each acute phase, 6-month and 12-month cytokine sample sets were statistically compared to healthy control CBA data using the non-parametric two-tailed Mann Whitney Test for unrelated samples.

Results

CHIKV resolution is associated with differential cytokine programs of increasing and decreasing trends

CHIKV causes a disease of crippling joint pain that has affected most of Asia and has demonstrated the capability to spread to non-tropical areas such as Europe and parts of North America [1]. Cytokines are inflammatory mediators and their balance is often associated with inflammatory disease [23]. Previously, the cytokine profiles of acute phase CHIKV patients have been examined [22]. Here we profiled cytokine levels in acute phase and 6- and 12-month follow-up CHIKV patient serum samples to determine a cytokine signature that may correlate with acute symptoms, following persistent joint pain and/or disease resolution. Blood samples were collected from 50 patients suffering from CHIKV infections during the 2007 Italian outbreak. Serum separated from whole blood was analyzed by cytokine bead analysis (CBA) for 13 cytokines with the intention of determining a cytokine profile during CHIKV acute phase and disease convalescence. Three cytokine profiles emerged from our data: decreasing, increasing and no-trend.

The first trend showed cytokine levels significantly higher in the acute samples compared to the follow-up time points revealing a decreasing pattern as patients left the acute phase. CXCL9/MIG (CXCL9), IL-6, CCL2/MCP-1(CXCL2) and CXCL10/IP-10 (CXCL10) cytokines had significantly decreased at both 6-month and 12-month follow-ups (Figure 1). Interestingly, some patients had extremely high levels in the acute phase; CXCL9 and CXCL10 reached similar levels as those of the community control levels (shown by dotted line). CCL2 levels decreased significantly lower than the control levels by 12 months. Taken together, this data demonstrated that CXCL9, IL-6, CCL2 and CXCL10 were initially increased with acute CHIKV infection and decreased over time.

The second cytokine trend that emerged described cytokines that significantly increased following the acute phase. Cytokine profiles that were markedly lower in the acute phase and subsequently increased included IL-1β, TNF-α, IL-12, IL-5, IL-10 and IFN-γ (Figure 2). The cytokine increase was more gradual than the previous decreasing trend, where fold changes were generally closer to 2. Both the 6- and 12-month follow-up were statistically increased compared to acute values for IL-5 levels. IL-1β, TNF-α, IL-12, IL-10 and IFN-γ had significantly increased by 12 months. Even though the average for these cytokines had also risen by 6 months it was not significant. Furthermore, the increasing trends for TNF-α, IL-5, and IL-10 reached similar levels to the community control levels (shown by dotted line) and IFN-γ reached significantly higher than controls at 12 months. Interestingly, although IL-1β and IL-12 increased through the observed time, these cytokines stayed significantly lower than those of the controls. This data showed that cytokines IL-1β, TNF-α, IL-12, IL-5, IL-10 and IFN-γ increased in the convalescence phase of CHIKV infection.

No significant change was seen for IL-2, IL-4, and IL-8 from the acute phase to the 12-month follow-up (Figure 3). Interestingly, IL-2 reached similar levels to those of the controls where IL-6 and IL-4 remained significantly raised and lowered, respectively.

CXCL10, CXCL9, IL-6 and IL-10 are possible biomarkers of virus, IgM and IgG levels in CHIKV patient serum

Previously, we have shown that the acute phase of West Nile Virus (WNV) can be described in 3 stages [24]. Since the samples from the CHIKV patients were taken at various stages of the acute phase, we went on to determine if there were cytokines marking the viral (V), IgM antibody initiation (AI) or seroconversion (SC) stage. Acute CHIKV patient samples were determined to be PCR positive for CHIKV (viral stage), IgM positive, IgG negative (IgM antibody initiation stage) or IgM positive, IgG positive (seroconversion stage). Samples were put into Viral stage (V), Antibody Initiation stage (AI) or Seroconversion stage (SC) according to the presence of CHIKV, IgM and IgG antibodies. Cytokine Bead Array analysis of the serum samples showed a significant decrease in CXCL10 and IL-10 from the viral stage to the seroconversion stage of the acute phase (Figure 4). The median of CXCL10 in the viral stage was approximately 7000 pg/ml and dropped to less than 1000 pg/ml after seroconversion. Interestingly, the IL-10 median decreased by 3 fold from the viral stage to the seroconversion stage.

Next we sought to determine if the high levels of IgG in the follow-up phases were also significantly associated with cytokine levels compared to the cytokine levels of patients with low levels of IgG. The patients were put into an IgG high group (H) or an IgG low group (L) and the levels of each cytokine were statistically compared for each group using the Mann Whitney U Test. In the 6-month follow-up phase CXCL9, CXCL10 and IL-6 were found to be statistically different between the high IgG group and the low IgG group (Figure 5A). High levels of all 3 cytokines were associated with high levels of IgG antibodies. IL-10 is also shown for comparison since it was statistically significant during the acute phase breakthrough and the 12-month follow-up. Interestingly, in the 12-month follow-up phase, CXCL9 was found to be statistically higher in the IgG high group where IL-10 was significantly lower in the high IgG group (Figure 5B). CXCL10 is shown for comparison at 12 months although it was not significantly different between the IgG high and IgG low groups. In summary, the results suggested that CXCL9, CXCL10 and IL-6 were associated with IgG levels in the 6-month follow-up phase and CXCL9 and IL-10 in the 12-month follow-up phase.
CXCL10, CXCL9 and IgG levels are possible biomarkers of CHIKV disease severity

IL-1β, IL-6 and RANTES were found to be associated with symptom severity of the Singapore 2007 CHIKV outbreak [22]. After determining the cytokine profiles of our Italian 2007 CHIKV patients during their disease resolution, we next sought to determine the association between symptom severity and cytokine levels. Patients were determined to be non-symptomatic (N), to have mild symptoms (M) or to have severe symptoms (S) depending on their responses to a questionnaire. The cytokine levels were then grouped by symptom level and a Mann-Whitney U test was used to determine significance among the severity groups for each cytokine. CXCL10 and CXCL9 were found to be significantly increased in the patients with mild and severe symptoms at 6 months following initial infection compared to the patients reporting no symptoms (Figure 6A). A 2 fold difference was seen between the medians of the non-symptomatic and severe patients for CXCL10 and a 5 fold difference for CXCL9. No statistical difference was seen for any of the 13 cytokines profiled for the 12-month follow-up. CXCL10 and CXCL9 at 12 months are shown for comparison (Figure 6B).

Moreover, we analyzed the IgG levels at the 6-month follow-up in patients with no symptoms, mild symptoms, and severe symptoms. The results showed IgG levels were statistically increased with...
Figure 2. CHIKV patient convalescence was associated with increasing levels of TNF-α, IL-5, IL-1β, IL-12, IFN-γ and IL-10. Cytokine Bead Array analysis of CHIKV patient serum samples showed that following the acute phase of CHIKV disease patients had increasing levels of TNF-α, IL-5, IL-1β, IL-12, IFN-γ and IL-10. Six-month and one-year cytokine levels were analysed for statistical significance using the Wilcoxon test for
symptom severity (Figure 6C). Taken together, these results suggested CXCL10, CXCL9 and IgG to be possible markers of CHIKV severity during early phases of disease resolution.

**Discussion**

CHIKV disease is a self-limiting disease caused by an *alphavirus* of the *Togaviridae* family. Although historically the virus only caused a disease of mild symptoms, a recent outbreak on La Reunion Island caused significant mortality due to genetic alterations [1,20,22,25,26]. Here we have investigated the immune response of an Italian population infected with the Indian Ocean genotype of CHIKV and have generated a cytokine signature for the initial infection to convalescence phase of CHIKV disease, the signature of viral and antibody production phases, and identified cytokines raised in patients with severe symptoms. We found that initial

**Figure 3.** IL-2, IL-8 and IL-4 were not associated with acute or convalescence phase of CHIKV disease. Cytokine Bead Array analysis of CHIKV patient serum samples showed that IL-2, IL-8 and IL-4 were not associated with acute phase or convalescence of CHIKV disease in patients. Six-month and one-year cytokine levels were analysed for statistical significance using the Wilcoxon test for Significance by comparing with acute phase values. Samples were analyzed for significance against healthy controls by the Mann-Whitney U test. The star symbol indicates a p-value (Mann-Whitney U test) less than 0.05 for 6- and 12-month groups compared to control values. The dotted line indicates the median of healthy control cytokine levels. Acute (A), 6-month follow-up (6), and 12-month follow-up (12).

doi:10.1371/journal.pntd.0001279.g002
infection and the subsequent convalescence were described by a set of decreasing and increasing cytokines. Furthermore, we have shown that CXCL10 and IL-10 levels were associated with the viral stage of the acute phase and CXCL10 and CXCL9 with high IgG levels of the 6-month follow-up. As well, CXCL10 and CXCL9 were markers of symptom severity. Importantly, these identified signatures depict the immunological programs and may be key to the development of therapeutics for the frequently re-emerging CHIKV disease.

Our analysis has identified 2 cytokine profiles that followed disease onset and continued with disease progression/convalescence. We found CXCL9, CXCL10, CCL2 and IL-6 levels were high in the acute phase and decreased as patients convalesced. Conversely, the trend for TNF-α, IL-1β, IL-2, IL-5 and IL-12 were low initially and increased as patients began to recover from acute illness. High levels of CXCL9, CXCL10, CCL2, and IL-6 in the acute phase possibly indicated an inflammatory program initiating adaptive T-cell immunity [27]. CXCL9 and CXCL10 are both chemokines induced by IFN-γ and are part of the chemokine program that regulates the migration of monocytes/macrophages, memory T cells and NK cells and are associated with the polarization of T cells [27]. IL-6, a pleiotropic cytokine, has a destructive role in rheumatoid arthritis (RA), contributing to joint inflammation as well as joint pain [28,29]. The increased IL-6 levels in the acute phase of our study may be the initiating factor of the severe joint pain symptoms reported in CHIKV patients which mimics RA. Furthermore, IL-6 is important during acute phases of the disease by acting as an important immune mediator of fever activating muscle metabolism to increase core body temperature [28]. Since fever is a common symptom of acute CHIKV disease, it is highly probable that the high IL-6 levels were contributing to the acute fever and the IL-6 decreasing trend followed patient core body temperature as it returned to resting temperature in the follow-up.

A second host immune response profile was characterized by TNF-α, IL-1β, IL-10, IL-12, IFN-γ and IL-5, which increased from...
Figure 5. The stages of CHIKV 6- and 12-month follow-up phases are marked by differentials in CXCL10, CXCL9, IL-6 and CXCL9, IL-10 respectively. IgG levels in 6- and 12-month patient serum samples were determined by ELISA. The samples were then grouped by IgG level; a
the acute phase into convalescence. Interestingly, TNF-α and IL-1β, which are known to co-induce the other’s expression, are both involved in chronic inflammatory diseases such as RA, chronic hepatitis B and C infection [23,29,30]. Importantly, TNF-α and IL-1β are main contributors to joint pain, which is the major symptom of RA. An internal balance of TNF-α or IL-1β levels is imperative as mis-regulation of either has been shown to be a major proponent of chronic diseases (RA). Our data indicated that these cytokines increased significantly during patient convalescence above those of the control group, but were not statistically changed in patients reporting mild or severe symptoms. These data may imply that the increased levels of TNF-α and IL-1β in the convalescence phase were not a major contributor of chronic damage causing persistent severe joint pain symptoms during the Italian outbreak even though these cytokines have previously been found to play a destructive role in chronic inflammatory diseases. Furthermore, TNF-α immunomodulators have previously been used as a common treatment for RA and IL-1β immunomodulators are effective with other chronic diseases such as systemic-onset juvenile idiopathic arthritis and in adult onset Still’s diseases [29,31]. Taken together, these findings suggest TNF-α and IL-1β therapies would not be effective controlling the prolonged symptoms of CHIKV disease since the raised levels during convalescence were not associated with patient severity.

Previously, cytokine profiles have been analyzed from patients during an Asian outbreak of CHIKV [22]. Ng and colleagues, investigated 30 cytokines and growth factors from 10 acute phase CHIKV patients, determined that the levels of 8 cytokines, 2 chemokines and 3 growth factors were significantly raised in patients compared to those of the control group. In accordance with their data, IL-6, CXCL9 and CXCL10 were also increased in the acute phase of our patients as compared to control. Since the results from the previous study did not follow the patients during convalescence, our study added significant insight to the progression of CHIKV disease. We found that these three cytokine levels decreased as the patients convalesced as discussed above. Conversely, we found CCL2 also to be increased in the acute phase compared to that of the control group, which was unchanged in the Ng study. Furthermore, Ng et al. found IL-5 and IL-10 were significantly increased in the acute phase where our data indicated that IL-5 and IL-10 were initially low and below control levels and increased following the acute phase. These discrepancies can possibly be explained by the patient populations: the Ng study patients and our patients were from significantly varied genetic backgrounds (Asian and European, respectively). Therefore, differences in immune response may reflect variations in immunological genetic programs. As well, the virus that caused the two outbreaks also differed genetically. Even though the virus that caused the Singapore outbreak was the same genotype as the Italian virus, the virus that infected the Italian patients had the A226V mutation in the E1 gene which was acquired on La Reunion Island [19]. Although the impact of this mutation has been shown to increase vector infectivity [20], the mutation has not been investigated in the human or mammalian immune response.

Previously, we have identified 3 stages of the acute phase of WNV; a viral stage, antibody initiation stage, and seroconversion stage [24]. As the viral load decreased in the WNV patients, IgM antibody levels were initiated and followed by IgM conversion to IgG, thereby marking the 3 stages of the acute phase. From this work we were able to identify the stages of the CHIKV patients and compare their respective serum cytokine levels. We found CXCL10 and IL-10 levels decreased as patients progressed through the viral stage to seroconversion. Since CXCL10 is often correlated with viral load, this observation was not surprising [32]. High IL-10 levels in the viral stage may act in an effort to control the IFN-γ program [33] shown by high CXCL10 levels. Furthermore, plasma levels of CCL2, IL-6 as well as CXCL10 have all been correlated with viral loads in virus infected individuals [24,34,35]. It is possible that the decrease of CCL2 and IL-6 we observed subsequent to the acute phase [14] correlated with viral clearance, although not with antibody levels. Analysis of the cytokine/antibody response was carried on to the 6- and 12-month follow-up where we grouped the patients on their IgG levels. CXCL9, CXCL10 and IL-6 were raised in the patients with increased IgG levels at 6 months and CXCL9 at the 12-month follow-up. These proinflammatory cytokine associations with high IgG levels may represent the persistence of an active immune system.

CXCL9 and CXCL10 as well as high IgG levels were found to be biomarkers of severe CHIKV symptoms. Previously, CXCL10 has been associated with severe viral disease supporting a role for CXCL10 in severe CHIKV disease [34–37]. These studies did not find an association with CXCL9 and severity as seen in our CHIKV patients. Our findings suggest high CXCL10 and CXCL9 associated with severity to be a unique signature of CHIKV. Interestingly, not only are CXCL10 and CXCL9 expressed in RA and other arthritis related disease patients [38–41], but they have been shown to be biomarkers for RA symptoms, implying a similar mechanism for joint destruction in CHIKV disease [42]. Moreover, CXCL9 and CXCL10 may be contributors of persistent immune activation in CHIKV disease leading to chronic symptoms, which implies cytokine immunomodulation may significantly improve patient treatment and recovery. Importantly, CXCL10 also has prognostic value in the treatment of viral hepatitis where CXCL10 levels follow disease recovery [43] supporting our finding and proposes a prognostic role for CXCL10 in CHIKV. Furthermore, our data puts forth CXCL10 and CXCL9 as possible drug targets for treatment of CHIKV symptoms in the convalescence phase due to the association with severity; however, further investigation is needed on CXCL10 and CXCL9 efficacy. In addition, not only are the identified cytokines useful as possible drug targets but the cytokine signatures described can also be applied when testing newly developed CHIKV therapeutics. As CHIKV therapeutics are evaluated in the CHIKV disease model, cytokine profiles can be used as an output for determining the efficacy of the novel therapeutics.

The synovial mast cell remains an important component during RA joint destruction by the exocytosis of intracellular granules containing inflammatory mediators. Currently, mast cell activation through FcγRs by high levels of circulating IgG antibodies is hypothesized to contribute to the pathological destruction of synovium in RA [44–46]. In addition, antibody immune complex formation within the joint stabilizing inflammatory mediators, such as chemokines and complement proteins, is another possible
Figure 6. CHIKV disease severity is associated with high CXCL10, CXCL9 and IgG levels at the 6-month time point. CHIKV patients were determined to be nonsymptomatic (N), to have mild symptoms (M) or to have severe symptoms (S). The cytokine and IgG levels were then grouped by symptom level and a Mann-Whitney U test was used to determine significance among the severity groups. CXCL10, CXCL9 and IgG were found to be significantly increased in the patients with mild and severe symptoms at 6 months following initial infection. The cross symbol indicates a p-value less than 0.05.

doi:10.1371/journal.pntd.0001279.g006
Inflammatory Cytokines Resolve Chikungunya Disease

The contribution of IgG levels and mast cells to CHIKV symptoms. Our findings support the need for further investigation into the facet of pathogenesis during RA [47,48]. We found high profiles as biomarkers for severity and symptom persistence. The cytokines investigated had one of two immunologically important profiles during CHIKV disease onset and convalescence; furthermore, CXCL10 and CXCL9 were found to be elevated in patients with CHIKV infection. The data presented here suggest that with further investigation, immunomodulators may significantly enhance patient recovery.

Acknowledgments
We wish to thank all the members of the Chikungunya Study Group of the Emilia Romagna region for their collaboration in the selection, follow-up and sampling of the patients evaluated in this study. We thank Stefania Varani for her academic and technical expertise.

Author Contributions
Conceived and designed the experiments: AAK DB DJK VS SR. Performed the experiments: DB SG PX AD AF IB. Analyzed the data: AAK MJC JFB-M. Contributed reagents/materials/analysis tools: GS MLN NS PG FC AP GR DJK VS SR. Wrote the paper: AAK.

References
1. Cavrini F, Gaibani P, Pierro AM, Rossini G, Landini MP, et al. (2009) Chikungunya: an emerging and spreading arthropod-borne viral disease. J Infect Dev Ctries 3: 745-753.
2. Sudeep AB, Parashar D (2008) Chikungunya: an overview. J Biosci 33: 443-449.
3. Demanou M, Antoniou-Nkondjo C, Ngapana E, Roussel D, Paupy C, et al. (2008) CHIKV outbreak in a rural area of Western Cameroon in 2006: A retrospective serological and entomological survey. BMC Res Notes 5: 293.
4. Niyas KP, Abraham R, Unnikrishnan RN, Mathew T, Nair S, et al. (2010) Molecular characterization of Chikungunya virus isolates from clinical samples and adult Aedes albopictus mosquitoes emerged from larvae from Kerala, South India. Virol J 7: 189. 1743-422X-7-189 [pii];10.1186/1743-422X-7-189 [doi].
5. Santhosh SR, Dash PK, Parida M, Khan M, Rao PV (2009) Appearance of E1: A296V mutant Chikungunya virus in Coastal Karnataka, India during 2008 outbreak. Virol J 6: 172. 1743-422X-6-172 [pii];10.1186/1743-422X-6-172 [doi].
6. (2011) NVBDCP (2007). Chikungunya fever situation in the country during 2006, http://nvbdcp.gov.in/Chikun-cases.html.
7. Parada P, de L, X, Jourdan J, Rovery C, Vaillant V, et al. (2006) Novel chikungunya virus variant in travelers returning from Indian Ocean islands. Emerg Infect Dis 12: 1499-1499.
8. Gibney KB, Fischer M, Prince HE, Kramer LD, St GK, et al. (2011) Chikungunya fever in the United States: a fifteen year review of cases. Clin Infect Dis 52: e121-e126. e124 [pii];10.1093/cid/cag124 [doi].
9. Mizuno Y, Kato Y, Takeshita N, Ujiie M, Kobayashi T, et al. (2010) Clinical and radiological features of imported chikungunya fever in Japan: a study of six cases at the National Center for Global Health and Medicine. J Infect Chemother 16: 1067-1073. 10.1016/j.jiac.2009.09.012 [pii];10.1016/j.jiac.2009.09.012 [doi].
10. Schuffenecker I, Iteman I, Michault A, Murri S, Frangeul L, et al. (2006) Persistence of proinflammatory response after severe respiratory pandemic influenza. Crit Care 13: R201. cc8208 [pii];10.1186/cc8208 [doi].
11. Thiboutot MM, Kammann S, Kawalekar OU, Shellock DJ, Khan AB, et al. (2010) Chikungunya virus: a potentially emerging epidemic? PLoS Negl Trop Dis 4: e787. 10.1371/journal.pntd.0000787 [pii];10.1371/journal.pntd.0000787 [doi].
12. Vazeille M, Moutailler S, Coudert D, Rousseau C, Khun H, et al. (2007) Two epidemic outbreak of Chikungunya virus infection in the Romagna region of Italy: a new perspective for the possible diffusion of tropical diseases in temperate areas? New Microbiol 31: 303-304.
13. Seyler T, Rizzo C, Finarelli AC, Po C, Alessio P, et al. (2008) Autochthonous and imported Italian chikungunya virus variant in travelers returning from Indian Ocean islands. Virol J 6: 199. 1743-422X-6-199 [pii];10.1186/1743-422X-6-199 [doi].
14. Sambri V, Cavrini F, Rossini G, Pierro A, Landini MP (2008) The 2007 summer 2007 outbreak. Euro Surveill 13. 10.2649/EuroSurf.2008.8.14 [pii];10.2649/EuroSurf.2008.8.14 [doi].
15. Liumbruno GM, Calteri D, Petropulacos K, Mattivi A, Po C, et al. (2008) The 2007 autochthonous and imported Italian chikungunya virus variant in travelers returning from Indian Ocean islands. Virol J 5: 33. 1743-422X-5-33 [pii];10.1186/1743-422X-5-33 [doi].
16. Varani S, Versaci MM, Cagno S, Schuster H, Lui S, et al. (2008) Interferon and interferon-induced chemokine expression is associated with control of acute viremia in West Nile virus-infected blood donors. J Infect Dis 198: 979-983. 10.1086/596132 [pii];10.1086/596132 [doi].
17. Bonilauri P, Bellini R, Calzolari M, Angelini R, Venturi L, et al. (2008) Chikungunya virus adapts to tiger mosquito via evolutionary convergence: a sign of seasonal synchronicity. Euro Surveill 13. 10.2649/EuroSurf.2008.7.8 [pii];10.2649/EuroSurf.2008.7.8 [doi].
18. Charrel RN, de L, X (2008) Chikungunya virus in north-eastern Italy: a consequence of seasonal synchrony. Euro Surveill 13. 10.2649/EuroSurf.2008.7.8 [pii];10.2649/EuroSurf.2008.7.8 [doi].
19. Chirathaworn C, Rattanatakkawit W, Poorowaran Y (2010) Serum IL-1β and IL-18BP levels in patients with Chikungunya virus infection. Virol J 7: 113. 10.1098/viur.2010.0077 [pii];10.1098/viur.2010.0077 [doi].
20. Ng LF, Chow A, Sun YJ, Kwek DJ, Lim PL, et al. (2009) IL-1β, IL-6, and RANTES as biomarkers of Chikungunya severity. PLoS One 4: e2616. 10.1371/journal.pone.0002616 [pii];10.1371/journal.pone.0002616 [doi].
21. Chirathaworn C, Rattanatakkawit W, Poorowaran Y (2010) Chikungunya: a potentially emerging epidemic? PLoS Negl Trop Dis 4: e1279. 10.1371/journal.pntd.00001279 [pii];10.1371/journal.pntd.00001279 [doi].
22. Ng LF, Chow A, Sun YJ, Kwek DJ, Lim PL, et al. (2009) IL-1β, IL-6, and RANTES as biomarkers of Chikungunya severity. PLoS One 4: e2616. 10.1371/journal.pone.0002616 [pii];10.1371/journal.pone.0002616 [doi].
23. Karløy HE, Lea SR, Preshaw PM, Taylor JJ (2007) The expanding family of interleukin-1 cytokines and their role in destructive inflammatory disorders. Clin Exp Immunol 149: 217-225. CED441 [pii];10.1111/j.1365-2249.2007.04341.x [doi].
24. Thiboutot MM, Kammann S, Kawalekar OU, Shellock DJ, Khan AB, et al. (2010) Chikungunya virus: a potentially emerging epidemic? PLoS Negl Trop Dis 4: e787. 10.1371/journal.pntd.0000787 [pii];10.1371/journal.pntd.0000787 [doi].
25. Sudeep AB, Parashar D (2008) Chikungunya: an overview. J Biosci 33: 443-449.
infection in ferrets. J Virol 82: 11308–11317. JVI.00691-08 [pii];10.1128/JVI.00691-08 [doi].

38. Hanaoka R, Kasama T, Muramatsu M, Yajima N, Shiozawa F, et al. (2003) A novel mechanism for the regulation of IFN-gamma-inducible protein-10 expression in rheumatoid arthritis. Arthritis Res Ther 5: R74–R81.

39. Ruschpler P, Lorenz P, Eichler W, Koczan D, Hanel C, et al. (2003) High CXCR3 expression in synovial mast cells associated with CXCL9 and CXCL10 expression in inflammatory synovial tissues of patients with rheumatoid arthritis. Arthritis Res Ther 5: R241–R252. 10.1186/ar783 [doi].

40. Ueno A, Yamamura M, Iwahashi M, Okamoto A, Aita T, et al. (2005) The production of CXCR3-agonistic chemokines by synovial fibroblasts from patients with rheumatoid arthritis. Rheumatol Int 25: 361–367. 10.1007/s00296-004-0449-x [doi].

41. Aggarwal A, Agarwal S, Misra R (2007) Chemokine and chemokine receptor analysis reveals elevated interferon-inducible protein-10 (IP-10)/CXCL10 levels and increased number of CCR5+ and CXCR3+ CD4+ T cells in synovial fluid of patients with enthesis-related arthritis (ERA). Clin Exp Immunol 149: 515–519. CEI1377 [pii];10.1111/j.1365-2249.2007.03377.x [doi].

42. Kuan WP, Tam LS, Wong CK, Ko FW, Li T, et al. (2010) CXCL9 and CXCL10 as Sensitive markers of disease activity in patients with rheumatoid arthritis. J Rheumatol 37: 257–264. jrheum.090769 [pii];10.3899/jrheum.090769 [doi].

43. Zeremski M, Markatou M, Brown QB, Dorante G, Cunningham-Rundles S, et al. (2007) Interferon gamma-inducible protein 10: a predictive marker of successful treatment response in hepatitis C virus/HIV-coinfected patients. J Acquir Immune Defic Syndr 45: 262–268. 10.1097/QAI.0b013e3180359219 [doi].

44. Nigrovic PA, Lee DM (2007) Synovial mast cells: role in acute and chronic arthritis. Immunol Rev 217: 19–37. IMR506 [pii];10.1111/j.1600-065X.2007.00506.x [doi].

45. Nigrovic PA, Lee DM (2005) Mast cells in inflammatory arthritis. Arthritis Res Ther 7: 1–11. ar1446 [pii];10.1186/ar1446 [doi].

46. Malbec O, Daeron M (2007) The mast cell IgG receptors and their roles in tissue inflammation. Immunol Rev 217: 206–221. IMR510 [pii];10.1111/j.1600-065X.2007.00510.x [doi].

47. Naandikumar KS, Holmdahl R (2006) Antibody-induced arthritis: disease mechanisms and genes involved at the effector phase of arthritis. Arthritis Res Ther 8: 223. ar2089 [pii];10.1186/ar2089 [doi].

48. Tsuibo N, Fernandez T, Li X, Nishi H, Cullere X, et al. (2011) Regulation of human neutrophil Fcgamma receptor IIa by C5a receptor promotes inflammatory arthritis in mice. Arthritis Rheum 63: 467–478. 10.1002/art.30141 [doi].