The Acute Phase of Chikungunya Virus Infection in Humans Is Associated With Strong Innate Immunity and T CD8 Cell Activation

Nadia Wauquier,[1,3,a] Pierre Becquart,[1,4,a] Dieudonné Nkoghe,[1] Cindy Padilla,[1] Angélique Ndjoyi-Mbiguino,[2] and Eric M. Leroy[1,4]

[1]Centre International de Recherches Médicales de Franceville, BP769, Gabon; [2]Université des Sciences de la Santé, Libreville, Gabon; [3]Institut National de la Santé et de la Recherche Médicale U543, Laboratoire d'Immunologie Cellulaire et Tissulaire, Université Pierre et Marie Curie Paris 6, Paris 75013, and [4]Institut de Recherche pour le Développement UMR224/MIVEGEC, 34394, Montpellier, France

Background. Rapidly spreading to new regions, including the islands of the Indian Ocean, Central Africa, and Europe, Chikungunya fever is becoming a major problem of public health. Unlike other members of the *alphavirus* genus, immune responses to Chikungunya virus (CHIKV) have been poorly investigated.

Methods. We conducted a large ex vivo multiplex study of 50 cytokine, chemokine, and growth factor plasma profiles in 69 acutely infected patients from the Gabonese outbreak of 2007. We also assessed a phenotypic study of T lymphocyte responses during human acute CHIKV infection.

Results. CHIKV infection in humans elicited strong innate immunity involving the production of numerous proinflammatory mediators. Interestingly, high levels of Interferon (IFN) α were consistently found. Production of interleukin (IL) 4, IL-10, and IFN-γ suggested the engagement of the adaptive immunity. This was confirmed by flow cytometry of circulating T lymphocytes that showed a CD8+ T lymphocyte response in the early stages of the disease, and a CD4+ T lymphocyte mediated response in the later stages. For the first time to our knowledge, we found evidence of CD95-mediated apoptosis of CD4+ T lymphocytes during the first 2 days after symptoms onset, ex vivo.

Conclusions. Together, our findings suggest that strong innate immunity is required to control CHIKV infection.

Chikungunya virus (CHIKV), an alphavirus of the *Togaviridae* family, is a small enveloped virus enclosing a single-stranded positive-sense RNA genome of approximately 12kb [1]. First isolated in 1953, CHIKV has since caused numerous outbreaks in Africa and Southeast Asia [1, 2]. In the last few years, CHIKV has spread to new regions of the world, including Indian Ocean islands [3–8], Central Africa [9–11] and even Europe [12]. A CHIKV outbreak in La Réunion island counted 266,000 clinical cases in 2005–2006 [3, 4], and at least 1.3 million cases were estimated in India in 2006–2007 [5–7]. Increased human mobility, spread of mosquito larvae through the international trade of used tires, and increased insecticide resistance have all contributed to the spread of CHIKV outbreaks. The very recent discovery of *Aedes albopictus*, a vector for CHIKV, and the first imported case of Chikungunya fever (CHIKF) in the south of France, as well as the recent outbreak in Italy in 2007, illustrate the potential for worldwide dissemination [12–14], making this virus a major threat to public health [15].

CHIKF is an acute illness. After a silent incubation period lasting 2–4 days, patients generally display an abrupt high fever, followed by skin rash and painful polyarthralgia. Symptoms may persist 3–7 days during the acute phase of the disease; however, chronic and incapacitating arthralgia can persist for months or even years [16]. Although CHIKF is not generally considered

Received 4 May 2010; accepted 29 September 2010; electronically published 14 December 2010.

Potential conflicts of interest: none reported.

[a]These authors contributed equally to this work.

Correspondence: Nadia Wauquier PhD, 769 CIRMF Franceville, Gabon (email: nadia.wauquier@gmail.com).

The Journal of Infectious Diseases 2011;204:115–123

0022-1899 (print)/1537-6613 (online)/2011/2041-0016$14.00
DOI: 10.1093/infdis/jiq006

life-threatening, severe forms involving neurological and hepatological complications can occur, and fatalities were recently reported [17, 18].

Previous studies have focused mostly on the mosquito vectors [13, 17, 19–26], the genotyping and molecular characterization of CHIKV isolates, and outbreak epidemiology [1, 4–6, 27]. Few studies have focused on CHIKF pathogenesis. Three studies have attempted to correlate cytokine and chemokine profiles with CHIKF disease characteristics [28–30]. For instance, severe CHIKF has been linked to increased plasma levels of proinflammatory interleukins IL-12 and IL-6, as well as decreased levels of proinflammatory chemokine RANTES [28, 30]. A few other cytokines and chemokines have also been found to be upregulated during CHIKV infection (IL-7, IL-12, IP-10, MCP-1, IFN-α, and IFN-γ), although only a limited number of patients have been studied and at a unique time point during the acute phase [28–31]. Somewhat contradictory results have been published regarding the cytokines IL-1β and IL-10 [28, 30]. Virtually nothing is known about the mounting of adaptive immune responses to CHIKV infection. T and B lymphocytes and monocyte-derived dendritic cells are not susceptible to CHIKV infection [32] but their role in the clearance of infected cells or possibly in the pathogenesis of the disease remains completely unestablished. Furthermore, although CHIKV is known to induce apoptosis in vitro [16], the expression of markers of apoptosis ex vivo is still undocumented.

Although the human immune response to CHIKV infection is poorly documented [28, 29, 32], the much more studied other members of the alphavirus genera bring some indications as to the possible interactions between CHIKV and the host's immune system. These studies show that the capacity of many alphaviruses to induce a severe disease is directly dependant on its ability to avoid or subvert the hosts' innate immunity. Type I Interferons (essentially IFN-α/β) are produced by most cell types and constitute the first line of defense against viral infections. Sindbis virus and Eastern equine encephalitis virus replication are strongly inhibited by IFN (types I and II) treatment [33–35], and in the absence of type I IFNs, the severity of Sindbis-induced disease is highly increased in animal models [33, 36]. A prominent role for type I IFNs in CHIKV clearance has also been suggested by both in vitro and animal models [32, 37].

Although the increasing impact of CHIKF in public health greatly exceeds that of other alphaviruses, immune responses to CHIKV infection have been neglected in comparison. Here, we conducted a large ex vivo study based on multiplex analysis of cytokine, chemokine, and growth factor plasmatic profiles of 69 CHIKF patients from the Gabonese outbreak of 2007. This is the largest cohort of CHIKF patients ever studied in this matter. Furthermore, we present phenotypic data on the T lymphocyte immune responses that occur during acute CHIKV infection in humans.

MATERIALS AND METHODS

Patients and Sample Preparation

A CHIKV outbreak occurred in Gabon, Central Africa, between March and August 2007. Centered in the capital Libreville, the outbreak spread to subsequent epidemic foci in several small towns along the route to the North of Gabon, generating approximately 20,000 cases. Between May and July 2007, 69 early blood samples, obtained during the first week after the onset of the disease, were collected from febrile patients who visited identified medical health-care centers in Libreville. Data on demographic characteristics, clinical features, and duration of symptoms of patients were collected. Blood was drawn into EDTA tubes (Becton Dickinson) and transported on ice daily to the laboratory of the Université des Sciences de Santé, Libreville. The tubes were centrifuged for 10 min at 2000*g* at room temperature. Plasma was recovered, aliquoted, and stored at −80°C. Isolation of peripheral blood mononuclear cells (PBMCs) was performed by standard histopaque density centrifugation if amount of blood was sufficient. Cells were suspended in fetal calf serum (FCS) (Invitrogen) with 10% dimethyl sulfoxyde (Merck) in Cryovial (Nunc), placed at −80°C overnight, then transferred to liquid nitrogen and transported to the Centre International de Recherches Médicales de Franceville.

Diagnosis of CHIKV infection was performed using the taqman quantitative real-time polymerase chain reaction (qRT-PCR) technology and specific primers and probes as previously described [11].

In addition, 30 plasma samples and 5 PBMC samples from randomly selected healthy volunteers (PCR for CHIKV negative, with no link with the outbreak) were used as controls. Control PBMC samples were collected from healthy volunteers from Franceville; control plasma samples were randomly selected among 4349 plasma samples collected from volunteers throughout Gabon.

Ethics

This study was approved by the Gabonese Ministry of Health (authorization N°000006 MSP/SG). Informed and written consent was obtained from all patients and healthy control subjects.

Multiplex Analysis

Levels of 50 cytokines, chemokines, and growth factors were measured in plasma samples using the Luminex technology (Bio-Rad). Two kits, the Bio-plex human cytokine 27-plex assay and the Bio-plex human cytokine 23-plex assay (Bio-Rad) (Table 1), were performed according to the manufacturer's instructions. Briefly, 25 μL of plasma samples was diluted 1:4 and incubated with anti-cytokine antibody-coupled beads for 1 hour. All incubations were performed at room temperature, and, between each step, complexes were washed 3 times in wash buffer (Bio-Rad) using a vacuum manifold. Beads were then incubated

Table 1. Investigated Cytokines, Chemokines, and Growth Factors

27-plex		23-plex	
Interleukin-1β	IL-1β	Interleukin-1α	Il-1α
Interleukin-1 receptor antagonist	IL-1RA	Interleukin-2 receptor α	IL-2Rα
Interleukin-2	IL-2	Interleukin-3	IL-3
Interleukin-4	IL-4	Interleukin-12p40	IL-12p40
Interleukin-5	IL-5	Interleukin-16	IL-16
Interleukin-6	IL-6	Interleukin-18	IL-18
Interleukin-7	IL-7	Cutaneous T-cell attracting chemokine	CTACK
Interleukin-8	IL-8	Growth-regulated oncogene-α	GRO-α
Interleukin-9	IL-9	Hepatocyte growth factor	HGF
Interleukin-10	IL-10	Intracellular adhesion molecule 1	ICAM-1
Interleukin-12p70	IL-12p70	Interferon-α2	IFN-α2
Interleukin-13	IL-13	Leukemia inhibitory factor	LIF
Interleukin-15	IL-15	Monocyte chemoattractant protein-3	MCP-3
Interleukin-17	IL-17	Macrophages colony-stimulating factor	M-CSF
Eotaxin	Eotaxin	Monokine induced by interferon-γ	MIG
Basic fibroblast growth factor	FGF-basic	Nerve growth factor-β	NGF-β
Granulocyte colony-stimulating factor	G-CSF	Stem cell factor	SCF
Granulocyte macrophage colony-stimulating factor	GM-CSF	Stem cell growth factor-β	SCGF-β
Interferon-γ	IFN-γ	Stromal cell-derived factor 1-α	SDF-1α
Interferon-inducible protein-10	IP-10	Tumor Necrosis Factor-β	TNF-β
Monocyte chemoattractant protein-1	MCP-1	Tumor-necrosis-factor related Apoptosis inducing ligand	TRAIL
Macrophage inflammatory protein-1α	MIP-1α	Vascular cell adhesion molecule-1	VCAM-1
Macrophage inflammatory protein-1β	MIP-1β		
Platelet-derived growth factor-ββ	PDGF-ββ		
Regulated-on-activation normal T-cell expressed and secreted	RANTES		
Tumor necrosis factor-α	TNF-α		
Vascular endothelial growth factor	VEGF		

with biotinylated detector antibody for 1 hour, before incubation with Streptavidin-phycoerythrin for 30 min. Finally, complexes were resuspended in 125 μL of detection buffer, and 200 beads were counted during acquisition on the Luminex 200 (Bio-Rad). Duplicates were performed; mean fluorescence intensity was analyzed and final concentrations were calculated in pg/mL.

Flow Cytometry

Patient's and control PBMC were thawed, washed thrice in RPMI 1640 with 1% penicillin/streptomycin, and resuspended at a final concentration of 2×10^6 cells/mL in RPMI with 10% FCS and 1% penicillin/streptomycin for 18 h at 37°C. Prior to staining, cells were washed in RPMI then distributed at a final concentration of 1×10^6 cells/mL in 0.1 mL of Isoflow (Beckman Coulter) in 9 tubes containing monoclonal antibody cocktails 1 to 9 (Table S1 of Supplementary Material). Cells were stained for 20 min at room temperature. After a final addition of 200 μL of Isoflow, 100,000 events were analyzed on FC500 cytometer (Beckman Coulter). Results were analyzed with CXP software (Beckman Coulter).

Statistical Analysis

Statistical analysis used the Student *t*-test or the Mann-Whitney Wilcoxon test to compare patients to controls. STATA software (version 9.0; Stata) was used, and a probability level of less than 0.05 was considered statistically significant.

RESULTS

Plasmatic Cytokine, Chemokine and Growth Factor Detection

We determined plasma cytokine profiles, using a multiplex-microbead immunoassay, in 69 patients with acute CHIKV infection (40% male; mean age, 27.8 years) and in 30 uninfected randomly selected volunteers (60% male; mean age, 47 years). Phylogenetic analysis showed that CHIKV isolates belonged to the Central-African lineage and harboured the A226V mutation [11]. Expression levels of 50 cytokines, chemokines, and growth factors were measured, and their kinetics were estimated with respect to the number of days between symptom onset and sampling. Seven patients were sampled on day 0 (D0, onset of

symptoms), 23 on D1, 21 on D2, 10 on D3, 2 on D4, 3 on D5 and 2 on D7.

To rule out any possible bias due to difference in age between both study groups, we randomly removed the older individuals and observed no significant differences in cytokine levels (data not shown).

No significant differences were found between patients and controls with respect to FGF-basic, GRO-α, IL-1β, IL-2, IL-5, IL-9, IL-10, IL-12p70, IL-15, IL-18, LIF, MIG, MIP-1α, TNF-α, and TRAIL levels (Figure S1 of the Supplementary Material).

The levels of 22 soluble proteins were significantly higher in CHIKF patients than in controls ($P < .05$), regardless of the sampling time (Figure 1). IL-6, IL-16, IL-17, IP-10, MCP-1, MIF, SDF-1α, IL-1ra, IL-2rα, G-CSF, GM-CSF, VEGF, IL-7, IL-12p40, and IFN-α2 were higher than the corresponding control values at every time point. PDGF-ββ and IL-4 were up-regulated on D0 and D1, SCGF-β on D2 and 3, IFN-γ on D1 to D4 (Figures 1*C*, 1*D*); pro-inflammatory chemokines MIP-1β and IL-8 were upregulated on D4 and D5, respectively (Figure 1*A*). Values of many cytokines showed strong inter-individual variability. For example, IFN-γ levels ranged from 10 to 600 pg/mL on D1, and IL-1RA levels ranged from 50 to 25 ng/mL. Levels of ICAM1, VCAM1, and RANTES were above the working range of the assay in all CHIKF patients but not in controls (data not shown). Interestingly, IFN-α2 levels were relatively homogenous, ranging from 400 to 700 pg/mL in CHIKF patients, and from 140 to 250 pg/mL in controls (Figure 1*D*).

In contrast, levels of IL-1α, MCP-3, HGF, M-CSF, β-NGF, SCF, CTACK, Eotaxin, IL-3, and TNF-β were significantly lower in patients than in controls (Figure 2) ($P < .05$).

Kinetics Analysis of Up-regulated Cytokines, Chemokines, and Growth Factors

We then examined kinetics among the significantly increased molecules (Figure 1). IL-2rα, IL-13, IFN-α2, and IL-4 were expressed at stable levels in CHIKF patients, from D0 to D7. In contrast, IL-16, IL-17, PDGF-ββ, IFN-γ and IL-7 were produced in higher quantities in patients in the first few days of the disease (D0, D1, and D2) and then decreased to control levels. IFN-γ levels peaked on D1 at a mean of 338 pg/mL. IP-10 levels peaked on D1 and then fell markedly on D2 but remained elevated (around 17 ng/mL) until symptom resolution. SCGF-β levels were similar to control values on D0 and D1, increased in most patients on D2 and D3, then returned to control values on D4 and D5. Levels of IL-6, IL-8, MCP-1, MIF, MIP-1β, SDF-1α, IL-1ra, G-CSF, GM-CSF, VEGF, and IL-12p40 rose toward the end of the first week after symptom onset (D4, D5, and D7).

Flow Cytometry Results

PBMC from 16 patients and 5 controls were studied for T lymphocyte surface markers. Three patients each were studied on days 0, 2, 3, and 4, and 4 patients on day 1. CD25, CD69, CD40L, CD28, CD29, CCR7, CCR5, CD95, and HLA-DR expression was analyzed, and results are presented as percentage of CD3+ CD4+ or CD3+ CD8+ cells. We were able to analyze absolute counts in 4 patients; all were lymphopenic (between 170 and 430 cells/mm^3; data not shown). However, CHIKF patients showed higher percentages of CD3+ CD8+ lymphocytes on D0, D1, and D2 compared to controls (Figure 3*A*). Percentage of CD8+ T cells peaked on D1 (40% versus 16% in controls) and remained elevated on D2. The percentage of CD3+ CD4+ lymphocytes in CHIKF patients was lower than in controls on the first 3 days after onset of symptoms and then rose to 46% on D3 (versus 40% in controls). These results were confirmed by a decrease in the T4/T8 ratio in the first 3 days of the disease, followed by a marked increase toward the end of the first week (Figure 4*B*). CD8+ lymphocyte activation was maximal by D1, as shown by increased expression of CD69 (47% versus 22% in controls) and HLA-DR (16% versus 7% in controls). The percentage of CD3+ CD4+ CD69+ was highest at 33% (versus 9% in controls), and that of CD3+ CD4+ HLA-DR+ at 6.5% (versus 3% in controls) by D4 (Figure 4*A*). Patients' CD3+ CD8+ T lymphocytes did not express CD95. CD3+ CD4+ T lymphocytes expressed CD95 on D0 and D1 (9.6% on D0 and 12.1% on D1, versus 2.5% in controls). Overall, no significant differences were observed in the expression of CD40L, CD28, CD29, CCR7, and CCR5 by CD4+ or CD8+ T lymphocytes in patients compared to controls (data not shown). Likewise, the CD3+ CD4+ CD25+ regulatory T cells percentages and activation status (CD69+ expression) were similar in the patients and controls (data not shown).

DISCUSSION

By following the kinetics of 50 plasmatic cytokines, chemokines, and growth factors at different stages of the disease, as well as a panel of cell membrane markers of activation, differentiation, and apoptosis of circulating T lymphocytes from a large number of patients in the first week of symptoms, we are now able to propose a model for human immune response during the acute phase of mild CHIKV infection. Our results indicate that CHIKV infection elicits strong innate responses, principally involving the production of antiviral IFN-α as previously shown, as well as many pro-inflammatory cytokines, chemokines, and growth factors. This was followed by the activation of the adaptive immunity through activation and proliferation of CD8+ T cells in the early stages of the disease. Evidence of CHIKV-induced CD95-mediated apoptosis of CD4+ T cells was also observed in the first 2 days of symptoms. Late stages of the acute phase were characterized by a classical switch to CD4+ T-cell response and the production of antiinflammatory proteins IL-1rα and IL-2RA.

Our most significant findings are the elevated levels of IFN-α and IFN-γ during acute CHIKV infection. IFN-α2 levels were

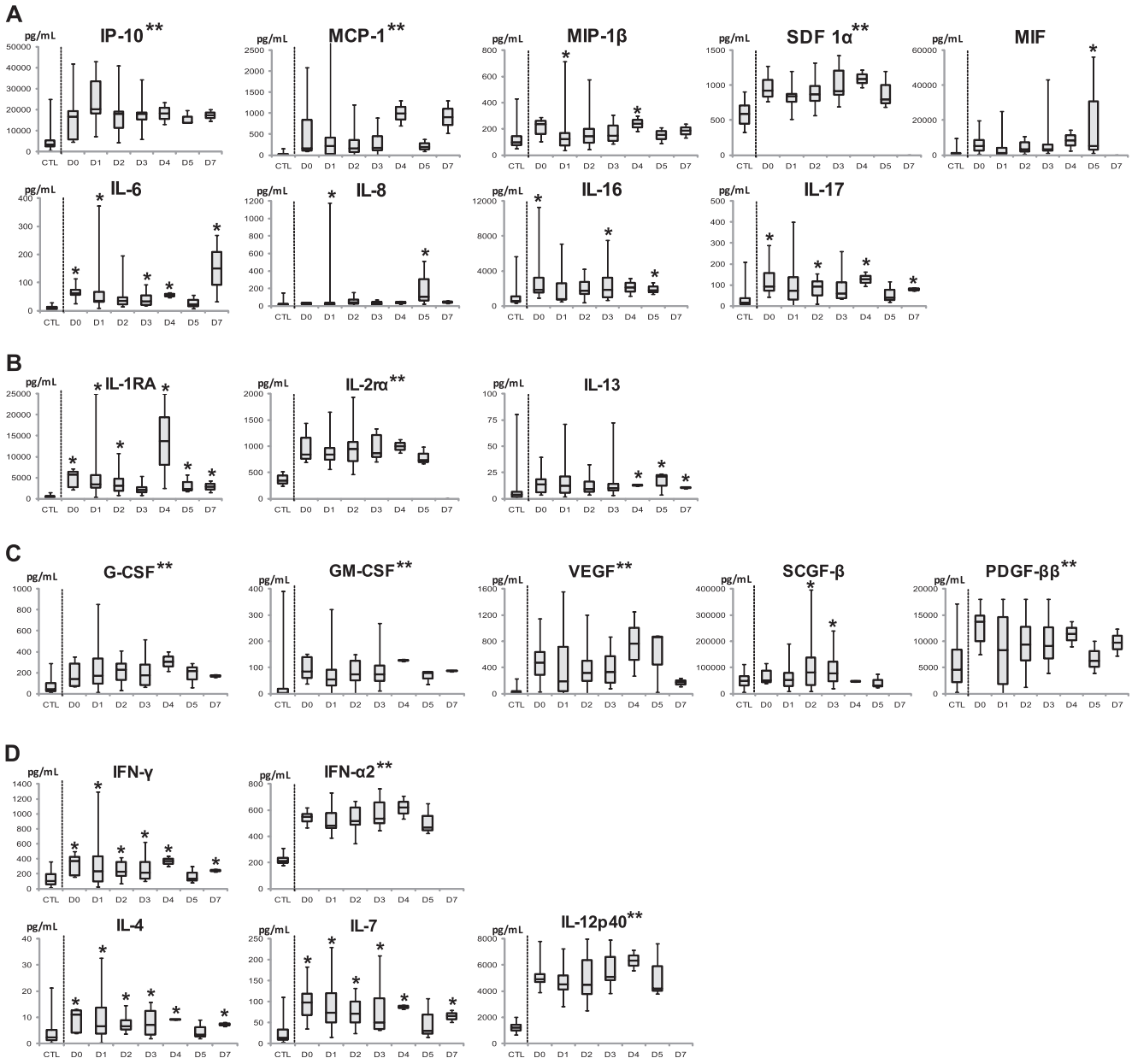

Figure 1. Box-and-whiskers representation of pro-inflammatory mediators (*A*), antiinflammatory cytokines (*B*), growth factors (*C*), and other cytokines (*D*) up-regulated in CHIKF patients' plasma. Levels are expressed in pg/mL, for controls (CTL) and patients according to the day of sampling after symptom onset (D0). The central horizontal line in the box marks the median of the samples. Box edges mark the first and third quartile (interquartile range within the box includes the central 50% of all values), and the whiskers show the maximum and minimum values. Student *t*-test or Mann Whitney Wilcoxon test were used to evaluate differences between controls and patients (*$P < .05$; **$P < .05$ at every time point).

high in all patients during the first week of symptoms, and IFN-γ levels were up-regulated in most patients in the first 3 days. When previously sought in clinically infected patients, levels of IFN-α were found slightly and highly increased [28, 31] and IFN-γ normal or marginally increased [28–30]. These discrepancies may easily be explained by the limited number of patients studied and differences in laboratory techniques. Contrary to what was recently shown [31], we found no correlation between viremia and levels of IFN-α. This may be due to several factors: absence of significant difference in the clinical forms of CHIKV infection observed in the infected patients, consistent and high levels of IFN-α among all patients, and bias due to the small size of some groups.

Interferons are important mediators of the immediate response to viral infections. There are 3 known classes of IFNs, designated types I, II, and III [38]. Type I IFNs comprise the IFN-α subtypes and IFN-β, which trigger signaling pathways inducing the expression of hundreds of interferon-stimulated

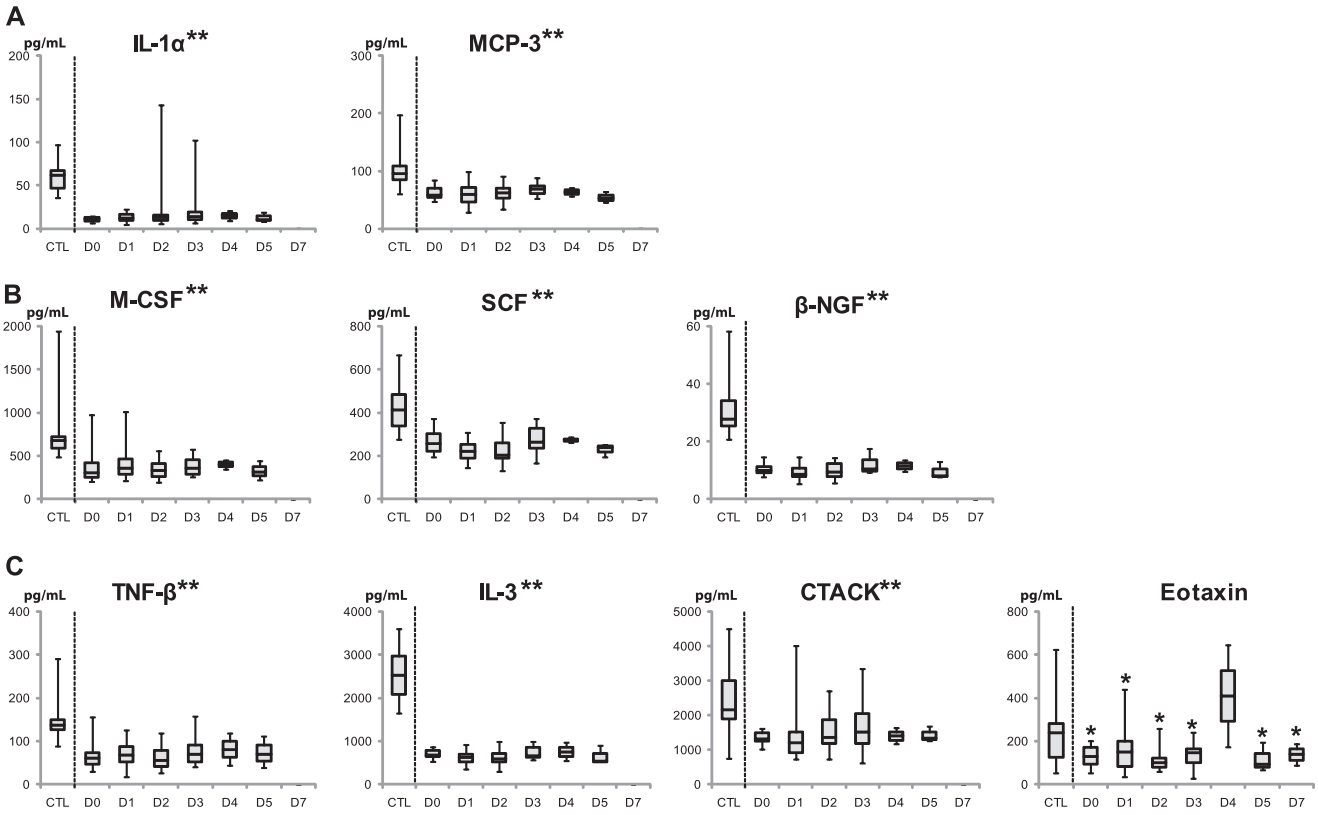

Figure 2. Box-and-whiskers representation of pro-inflammatory chemokines (*A*), growth factors (*B*), cytokines and chemokines of the adaptive immunity (*C*) down-regulated in CHIKF patients' plasma. Levels are expressed in pg/mL, for controls (CTL) and patients according to the day of sampling after symptom onset (DO). The central horizontal line in the box marks the median of the samples. Box edges mark the first and third quartile (interquartile range within the box includes the central 50% of all values) and the whiskers show the maximum and minimum values. Student *t*-test or Mann Whitney Wilcoxon test were used to evaluate differences between controls and patients (*$P < .05$; **$P < .05$ at every time point).

genes resulting in the synthesis of multiple antiviral proteins and the establishment of an overall antiviral state. Type II IFN, or IFN-γ, is secreted mostly by activated NK cells and T lymphocytes, rather than in direct response to viral infection. A prominent role for interferons in the inhibition of CHIKV replication has been strongly suggested by several in vitro or animal model studies. For example, pretreatment of cultured cells with IFN-α, -β, or -γ inhibited CHIKV replication in a dose-dependent manner [31, 32], and a mouse model with an abrogated IFN-α/β signaling pathway developed severe CHIKV infection [37]. High levels of IFN-α were produced after in vitro infection of monocytes [39], but a recent study suggests that fibroblasts could be the principal source of type I IFNs during CHIKV infection [31]. Interferons, and especially the type I IFN system, appear to play a primary role in controlling the severity of many viral infections including alphaviral infections [38, 40]. Severe alphavirus infection is associated with the absence or downregulation of IFN type I responses [36], whereas strong IFN responses seem to protect the host and allow only mild infections [34, 35]. The importance of IFNs is further supported by the fact that alphaviruses appear to have evolved to suppress host IFN response. Indeed, the nonstructural protein 2 of Sindbis virus and Semliki Forest virus, and the capsid protein of Venezuelan equine encephalitis virus and Eastern equine encephalitis virus have been shown to directly induce transcriptional shutoff in infected cells, thereby inhibiting the IFN response [41–43]. Our results suggest that during typical CHIKV infection, strong IFN-α production could be crucial in the rapid control of the viremia. In this respect, it would be interesting to investigate IFN responses in the recently described severe cases of CHIKF [18].

Our study shows that CHIKV infection induces a strong inflammatory response that seems to be orchestrated at first by the production of IL-16, IL-17, MCP-1, IP-10, not to mention that a few patients expressed very high levels of MIP-1α. The end of the acute phase was characterized by the production of pro-inflammatory MIF, MIP-1β, SDF-1α, and IL-6 and IL-8. RANTES levels were also high in all patients during the first week after symptom onset. RANTES, MCP-1, IP-10, MIP-1β, and IL-8 are produced by activated macrophages that are susceptible to CHIKV infection [32]. These chemokines play a major role in recruiting leucocytes to sites of infection, orchestrating the deployment of efficient antiviral defenses. Although variations between patients were observed, no significant differences were noted between CHIKF patients and controls in

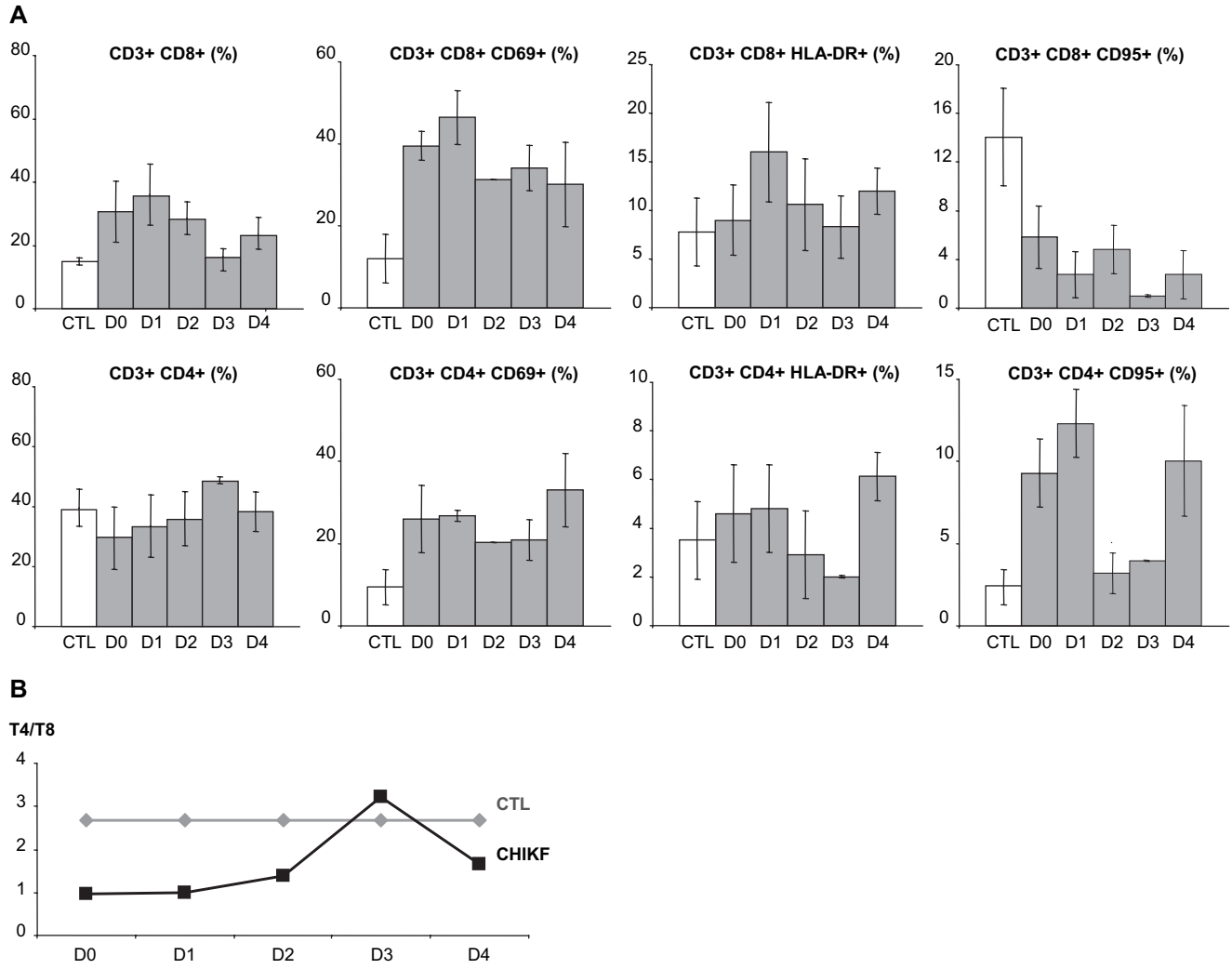

Figure 3. Cellular responses to CHIKV infection. Percentages of indicated lymphocyte populations among PBMC of controls (*white bars*) and CHIKF patients (*gray bars*). Patients are separated according to the day of sampling after symptom onset (DO) (*A*). T4/T8 ratio in controls (*gray line*) and CHIKF patients (*black line*) (*B*). Patients are separated according to the day of sampling after symptom onset (DO).

the production of pro-inflammatory MIG. Inflammatory response has been studied in other alphaviral infections. IL-6 up-regulation in CHIKF is consistent with data obtained in mice infected with molecular clones of Venezuelan equine encephalitis virus and Sindbis virus [44, 45]. In vitro infection of fibroblasts and macrophages by Ross River Virus (RRV), an alphavirus that upon human infection generates similar symptoms to CHIKF, elicits IL-8 and MCP-1 expression [46]. Neutralization of these factors attenuates RRV disease in mice, suggesting that their oversecretion may be involved in the pathogenesis of RRV-associated arthritis [47]. It would be interesting to study the roles of IL-8 and MCP-1 in CHIKV pathogenesis. The strong initial inflammatory response that we observed in CHIKF patients was followed by the production of antiinflammatory molecules IL-1RA in the late stages of the disease. That could limit bystander damages that would otherwise be caused by a prolonged inflammatory state.

High plasma levels of IFN-γ, IL-4, IL-7, and IL-12p40, cytokines that promote the adaptive immunity, suggested the involvement of cellular responses. Flow cytometry confirmed the mounting of rapid cellular responses as described in the very recent study of a cohort of patients from La Reunion Island [30], with an up-regulation of CD3+ CD8+ cells, and a down-regulation of CD3+ CD4+ cells in the first 2 days of symptoms. In RRV infection, predominant CD8+ T lymphocytes are associated with rapid recovery, whereas predominant CD4+ T cell responses are associated with chronic forms [48]. Activated, cytotoxic lymphocytes might therefore play an important part in the clearance of CHIKV infected cells. A key role for natural killer (NK) cells in the clearance of infected cells and in the development of CHIKV arthralgia has also been suggested [28]. However, we do not confirm the upregulation of NK activating IL-15 previously observed [28]. The B cell-promoting cytokines IL-4 and in some cases IL-10, were also upregulated in the first few days after

symptom onset probably initiating the production of CHIKV-specific IgG. Furthermore, CD4+ T lymphocytes, which are also involved in the promotion of humoral responses, were strongly activated toward the end of the acute phase. Interestingly, the percentage CD95+ expression by these cells was elevated on days 0 and 1, suggesting early apoptosis of CD4+ T lymphocytes. Infection with CHIKV is known to directly induce apoptosis of epithelial cells in vitro [32]. Our results suggest that CHIKV could also indirectly trigger apoptosis of CD4+ T lymphocytes ex vivo, and this occurs through CD95/CD95L interaction. During viral infections, CD95/CD95L apoptosis, especially of restimulated CD4+ cells, participates in the regulation of the intensity and the duration of cellular immune responses [49]. Furthermore, intense lymphopenia in CHIKV patients has been previously described, and CD95 apoptosis of CD4+ T cells could account for at least part of this phenomenon [50].

This report is the first to study such a large cohort of CHIKF patients and by so widely contributes to the better understanding of the kinetics of human immune responses to CHIKV infection. Our results suggest that innate immune response, involving antiviral cytokines and a strong pro-inflammatory response, as well as CD8+ cytotoxic T cells, play a key role in the primary human response to acute CHIKV infection in humans. The absence or deregulation of one of these mechanisms could be accounted for the more severe cases of CHIKF.

Supplementary Data

Supplementary material are available online at http://jid.oxfordjournals.org/.

Funding

This work was supported by a special Crédits incitatifs grant from the Institut pour la Recherche et le Développement (IRD) and a grant from Total-Gabon. CIRMF is supported by the Government of Gabon, Total-Fina-Elf Gabon, and the Ministère des affaires Etrangères, France. This work also received a grant from Agence National pour la Recherche (ANR), appel MIME 2006, number ANR-06-MIME-022-01.

Acknowledgments

We thank P. Yaba, A. Délicat, P. Ngari from Centre International de Recherches Médicales de Franceville, Gabon, for technical assistance during this work. We are grateful to the laboratory of virology of the faculty of medicine of Libreville for hosting us. We are also very grateful to the Gabonese Ministry of Health (especially Dr Obame-Edou and Dr Toung-Mve) and the healthcare workers of medical centres in Libreville, for their help in obtaining the samples.

References

1. Powers AM, Logue CH. Changing patterns of chikungunya virus: reemergence of a zoonotic arbovirus. J Gen Virol **2007**; 88:2363–2377.
2. Robinson MC. An epidemic of virus disease in Southern Province, Tanganyika Territory, in 1952-53. I. Clinical features. Trans R Soc Trop Med Hyg **1955**; 49:28–32.
3. Paquet C, Quatresous I, Solet JL, et al. Chikungunya outbreak in Reunion: epidemiology and surveillance, 2005 to early January 2006. Euro Surveill **2006**; 11:E060202 3.
4. Bessaud M, Peyrefitte CN, Pastorino BA, et al. Chikungunya virus strains, Reunion Island outbreak. Emerg Infect Dis **2006**; 12:1604–1606.
5. Schuffenecker I, Iteman I, Michault A, et al. Genome microevolution of chikungunya viruses causing the Indian Ocean outbreak. PLoS Med **2006**; 3:e263.
6. Ravi V. Re-emergence of chikungunya virus in India. Indian J Med Microbiol **2006**; 24:83–84.
7. Saxena SK, Singh M, Mishra N, Lakshmi V. Resurgence of chikungunya virus in India: an emerging threat. Euro Surveill **2006**; 11:E060810 2.
8. Mavalankar D, Shastri P, Raman P. Chikungunya epidemic in India: a major public-health disaster. Lancet Infect Dis **2007**; 7:306–307.
9. Muyembe-Tamfum JJ, Peyrefitte CN, Yogolelo R, et al. [Epidemic of Chikungunya virus in 1999 and 2000 in the Democratic Republic of the Congo]. Med Trop (Mars) **2003**; 63:637–638.
10. Pastorino B, Muyembe-Tamfum JJ, Bessaud M, et al. Epidemic resurgence of Chikungunya virus in democratic Republic of the Congo: identification of a new central African strain. J Med Virol **2004**; 74:277–282.
11. Leroy EM, Nkoghe D, Ollomo B, et al. Concurrent chikungunya and dengue virus infections during simultaneous outbreaks, Gabon, 2007. Emerg Infect Dis **2009**; 15:591–593.
12. Rezza G, Nicoletti L, Angelini R, et al. Infection with chikungunya virus in Italy: an outbreak in a temperate region. Lancet **2007**; 370:1840–1846.
13. Moutailler S, Barre H, Vazeille M, Failloux AB. Recently introduced *Aedes albopictus* in Corsica is competent to Chikungunya virus and in a lesser extent to dengue virus. Trop Med Int Health **2009**; 14:1105–1109.
14. ProMED-mail. Chikungunya (08): France ex Singapore.: ProMED-mail **2009.**
15. Charrel RN, de Lamballerie X, Raoult D. Chikungunya outbreaks–the globalization of vectorborne diseases. N Engl J Med **2007**; 356:769–771.
16. Brighton SW, Prozesky OW, de la Harpe AL. Chikungunya virus infection: a retrospective study of 107 cases. S Afr Med J **1983**; 63:313–315.
17. Pialoux G, Gauzere BA, Jaureguiberry S, Strobel M. Chikungunya, an epidemic arbovirosis. Lancet Infect Dis **2007**; 7:319–327.
18. Simon F, Tolou H, Jeandel P. [The unexpected Chikungunya outbreak]. Rev Med Interne **2006**; 27:437–441.
19. Jupp PG, McIntosh BM. *Aedes furcifer* and other mosquitoes as vectors of chikungunya virus at Mica, northeastern Transvaal, South Africa. J Am Mosq Control Assoc **1990**; 6:415–420.
20. Delatte H, Paupy C, Dehecq JS, Thiria J, Failloux AB, Fontenille D. [*Aedes albopictus*, vector of chikungunya and dengue viruses in Reunion Island: biology and control]. Parasite **2008**; 15:3–13.
21. Paupy C, Ollomo B, Kamgang B, et al. Comparative role of *Aedes albopictus* and *Aedes aegypti* in the emergence of dengue and Chikungunya in Central Africa. Vector Borne Zoonotic Dis **2009.**
22. de Lamballerie X, Leroy E, Charrel RN, Ttsetsarkin K, Higgs S, Gould EA. Chikungunya virus adapts to tiger mosquito via evolutionary convergence: a sign of things to come? Virol J **2008**; 5:33.
23. Paupy C, Delatte H, Bagny L, Corbel V, Fontenille D. *Aedes albopictus*, an arbovirus vector: from the darkness to the light. Microbes Infect **2009**; 11:1177–1185.
24. Jupp PG, McIntosh BM, Dos Santos I, DeMoor P. Laboratory vector studies on six mosquito and one tick species with chikungunya virus. Trans R Soc Trop Med Hyg **1981**; 75:15–19.
25. Reiter P, Fontenille D, Paupy C. *Aedes albopictus* as an epidemic vector of chikungunya virus: another emerging problem? Lancet Infect Dis **2006**; 6:463–464.
26. Ng LC, Tan LK, Tan CH, et al. Entomologic and virologic investigation of Chikungunya, Singapore. Emerg Infect Dis **2009**; 15:1243–1249.
27. Sam IC, Chan YF, Chan SY, et al. Chikungunya virus of Asian and Central/East African genotypes in Malaysia. J Clin Virol **2009**; 46:180–183.

28. Ng LF, Chow A, Sun YJ, et al. IL-1beta, IL-6, and RANTES as biomarkers of Chikungunya severity. PLoS One **2009**; 4:e4261.
29. Lee N, Wong CK, Lam WY, et al. Chikungunya fever, Hong Kong. Emerg Infect Dis **2006**; 12:1790–1792.
30. Hoarau JJ, Jaffar Bandjee MC, Trotot PK, et al. Persistent chronic inflammation and infection by Chikungunya arthritogenic alphavirus in spite of a robust host immune response. J Immunol **2010**; 184:5914–5927.
31. Schilte C, Couderc T, Chretien F, et al. Type I IFN controls chikungunya virus via its action on nonhematopoietic cells. J Exp Med **2010**; 207:429–442.
32. Sourisseau M, Schilte C, Casartelli N, et al. Characterization of reemerging chikungunya virus. PLoS Pathog **2007**; 3:e89.
33. Ryman KD, Klimstra WB, Nguyen KB, Biron CA, Johnston RE. Alpha/beta interferon protects adult mice from fatal Sindbis virus infection and is an important determinant of cell and tissue tropism. J Virol **2000**; 74:3366–3378.
34. Zhang Y, Burke CW, Ryman KD, Klimstra WB. Identification and characterization of interferon-induced proteins that inhibit alphavirus replication. J Virol **2007**; 81:11246–11255.
35. Gardner CL, Yin J, Burke CW, Klimstra WB, Ryman KD. Type I interferon induction is correlated with attenuation of a South American eastern equine encephalitis virus strain in mice. Virology **2009**; 390:338–347.
36. Ryman KD, Meier KC, Gardner CL, Adegboyega PA, Klimstra WB. Non-pathogenic Sindbis virus causes hemorrhagic fever in the absence of alpha/beta and gamma interferons. Virology **2007**; 368:273–285.
37. Couderc T, Chretien F, Schilte C, et al. A mouse model for Chikungunya: young age and inefficient type-I interferon signaling are risk factors for severe disease. PLoS Pathog **2008**; 4:e29.
38. Randall RE, Goodbourn S. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. J Gen Virol **2008**; 89:1–47.
39. Her Z, Malleret B, Chan M, et al. Active infection of human blood monocytes by chikungunya virus triggers an innate immune response. J Immunol **2010**; 184:5903–5912.
40. Ryman KD, Klimstra WB. Host responses to alphavirus infection. Immunol Rev **2008**; 225:27–45.
41. Frolova EI, Fayzulin RZ, Cook SH, Griffin DE, Rice CM, Frolov I. Roles of nonstructural protein nsP2 and alpha/beta interferons in determining the outcome of Sindbis virus infection. J Virol **2002**; 76:11254–11264.
42. Garmashova N, Gorchakov R, Volkova E, Paessler S, Frolova E, Frolov I. The Old World and New World alphaviruses use different virus-specific proteins for induction of transcriptional shutoff. J Virol **2007**; 81:2472–2484.
43. Breakwell L, Dosenovic P, Karlsson Hedestam GB, et al. Semliki Forest virus nonstructural protein 2 is involved in suppression of the type I interferon response. J Virol **2007**; 81:8677–8684.
44. Grieder FB, Davis BK, Zhou XD, Chen SJ, Finkelman FD, Gause WC. Kinetics of cytokine expression and regulation of host protection following infection with molecularly cloned Venezuelan equine encephalitis virus. Virology **1997**; 233:302–312.
45. Klimstra WB, Ryman KD, Bernard KA, Nguyen KB, Biron CA, Johnston RE. Infection of neonatal mice with sindbis virus results in a systemic inflammatory response syndrome. J Virol **1999**; 73:10387–10398.
46. Mateo L, La Linn M, McColl SR, Cross S, Gardner J, Suhrbier A. An arthrogenic alphavirus induces monocyte chemoattractant protein-1 and interleukin-8. Intervirology **2000**; 43:55–60.
47. Lidbury BA, Rulli NE, Suhrbier A, et al. Macrophage-derived proinflammatory factors contribute to the development of arthritis and myositis after infection with an arthrogenic alphavirus. J Infect Dis **2008**; 197:1585–1593.
48. Fraser JR, Becker GJ. Mononuclear cell types in chronic synovial effusions of Ross River virus disease. Aust N Z J Med **1984**; 14:505–506.
49. Ju ST, Panka DJ, Cui H, et al. Fas(CD95)/FasL interactions required for programmed cell death after T-cell activation. Nature **1995**; 373:444–448.
50. Staikowsky F, Talarmin F, Grivard P, et al. Prospective study of Chikungunya virus acute infection in the Island of La Reunion during the 2005-2006 outbreak. PLoS One **2009**; 4:e7603.