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Persistent Chronic Inflammation and Infection by Chikungunya Arthritogenic Alphavirus in Spite of a Robust Host Immune Response

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Alphaviruses, including Chikungunya virus (CHIKV), produce a transient illness in humans, but severe forms leading to chronic incapacitating arthralgia/arthritis have been reported by mechanisms largely ill-characterized. The pathogenesis of CHIKV was addressed in a prospective cohort study of 49 hospitalized patients from Reunion Island subsequently categorized into two distinct groups at 12 mo postinfection. Comprehensive analyses of the clinical and immunological parameters throughout the disease course were analyzed in either the “recovered” or the “chronic” groups to identify prognostic markers of arthritis-like pathology after CHIKV disease. We found that the chronic group consisted mainly of more elderly patients (>60 y) and with much higher viral loads (up to 10¹⁰ viruses per milliliter of blood) during the acute phase. Remarkably, a rapid innate immune antiviral response was demonstrated by robust dendritic/NK/CD4/CD8 cell activation and accompanied by a rather weak Th1/Th2 cytokine response in both groups. Interestingly, the antiviral immune response witnessed by high levels of IFN-α mRNA in PBMCs and circulating IL-12 persisted for months only in the chronic group. CHIKV (RNA and proteins) was found in perivascular synovial macrophages in one chronic patient 18 mo postinfection surrounded by infiltrating NK and T cells (CD4⁺⁺ but rare cytotoxic CD8⁺). Fibroblast hyperplasia, strong angiogenesis, tissue lesions given the high levels of matrix metalloproteinase 2, and acute cell death [high cleaved poly (ADP-ribose) polymerase staining] were observed in the injured synovial tissue. These observed cellular and molecular events may contribute to chronic arthralgia/arthritis targeted by methotrexate used empirically for effective treatment but with immunosuppressive function in a context of viral persistence. The Journal of Immunology, 2010, 184: 5914–5927.

Arthropiruses are globally distributed and are maintained in nature by continuous cycles of transmission between mosquitoes and vertebrate hosts, such as mammals (for review, see Refs. 1–3). Bites by infected mosquitoes can result in epidemic infections of humans, with most cases occurring after seasonal rains when mosquito numbers are high. Human diseases and pathological mechanisms caused by the African and Asian arthritogenic alphaviruses have not been extensively studied because they largely occur in underdeveloped or developing countries, where they often do not represent a major health priority.

Chikungunya virus disease (CHIKVD), which has affected as many as 3–4 million people in the Indian Ocean zone and spread to continental Europe in 2005–2007, has received recently considerable attention, and many parallels may be drawn between CHIKVD and the other alphaviral arthritides, such as Ross River virus (RRV) disease (4–10). Classically, infected people adopt a characteristic stooped walking position, which is the hallmark of the disease and from which it derives its name “Chikungunya,” meaning “to walk bent over,” in the Kimakonde language of Mozambique. The classic clinical symptoms are abrupt febrile...
illness (temperature usually \(>38.9^\circ\)C), polyarthralgia (\(>90\)% of cases), and maculopapular rash (a form of microvasculitis, more than one third of cases). The pain can be excruciating and is usually symmetrical and involved more than one joint. Fingers, wrists, elbows, toes, ankles, and knees are most commonly affected as described for most of the alphaviruses (4). Previously injured joints are especially susceptible. Paresthesia (numbness and tingling) in the skin covering affected joints has also been described, although the joints are often swollen there are usually no other signs of inflammation (11, 12). The other symptoms include myalgia, headache, edema of the extremities, and gastrointestinal complaints (11–13). The incubation period for CHIKV ranges from 3 to 7 d, in agreement with data from other alphaviruses (14), and as few as 5% of asymptomatic CHIKV cases were generally reported.

Remarkably, the epidemic of CHIKV in La Réunion Island in a nonimmune population has revealed exceptional forms of the disease (e.g., mother-to-child transmission associated with encephalitis and deaths and extensive bullous eruption) and severe complications in adults, such as persistent arthralgia, destructive arthritis, hepatitis, autoimmune neurologic pathologies (Guilain Barré), cardiologic manifestations, and deaths (7, 9, 15–20).

The CHIKV infection is characterized by a strong viremia (10^5 up to 10^12 viruses per milliliter of blood as established by real-time PCR techniques) (6), inducing an acute disease and an immune response that should, in principle, limit the duration of the infection. High viral load is more likely to be detected in newborns and elderly CHIKV disease patients, but to what extent can this contribute to increased pathogenesis remains to be addressed (21). Interestingly, and in contrast to Dengue virus, the apparition of IgG anti-CHIKV Abs was detected in the first week (even at day 2 in some patients) following infection and illustrating the rapid seroconversion and robust adaptive immune response (8). Clinical biological laboratory parameters (e.g., acute phase proteins, such as C-reactive protein [CRP] and complement) remained largely within the normal ranges, although major lymphopenia and moderate thrombocytopenia characterized the acute infectious phase of CHIKV fever (11, 12, 22).

The acute signs and symptoms usually resolve in less than 2 wk, but arthralgia may linger for weeks, months, or even years, and this is a clinical sign that may distinguish CHIKV from Dengue virus infection. Typically, the joint symptoms occurred in a fluctuating manner but did not change anatomical location. Chronic incapacitating arthritis has already been described for several alphaviruses (1, 2, 4, 23), and rheumatic manifestations in 10–20% of the CHIKV patients typically consisted of a febrile arthritis mainly affecting the extremities (ankles, wrists, or phalanges) (11, 16, 20, 24–26). Pain within or around tendons was also a common trait and evolving to tenosynovitis or enthesopathy but rarely to joint synovitis by ill-characterized mechanisms. Recently, Chopra et al. (27) reported high levels of CHIKV IgM in a cohort of Indian patients with post-CHIKV rheumatoid arthritis (RA)-like illnesses. These patients were clearly naive for musculoskeletal disorders prior to CHIKV infection. Over 90 down to 60% of the patients were IgM* in a period of 30–180 d postinfection (PI), and interestingly, 5–10% of patients with CHIKV arthritis were also positive for rheumatoid factor and anti-cyclic citrullinated peptide. This study did not reveal any major RA classic erosions of the cartilage and bones, and hence the post-CHIKV RA is reminiscent of but distinguishable from autoimmune RA. Some inflammatory processes were suggested by high levels of CRP (in >70% of the cohort, mean of 15 mg/l) and necessitating effective treatment regimes, such as hydroxychloroquine, methotrexate, sulfasalazine, and some corticoids, for a period of up to 6 mo (27). The persistence of the specific IgM response months after the initial infection has already been observed for several alphaviruses and may be related to viral persistence but through poorly understood mechanisms (28).

The host response to viral infection represents a complex orchestration of divergent pathways designed to eradicate the virus and benefit the host. However, many pathways that are involved in antiviral defense can also have untoward effects on the host, including NK cell and CTL hyperresponses, cytokine hyperresponses, and severe apoptosis, resulting in either dysfunction or death of infected or neighboring uninfected cells. Thus, the most effective defense response needs to be sufficiently lethal to rapidly kill invading pathogens, but it is essential, at the same time, to avoid collateral damage to the host by anti-inflammatory mechanisms, which remain largely ill-characterized in CHIKVD.

Alternately, virus persistence could be linked to immune inefficacy, viral escape phenomenon, or both. Understanding to what extent protective and pathogenic immune mechanisms are present in CHIKVD and contributing to chronic arthralgia/arthritis is critical to have a better insight into the pathology and possible therapeutic routes targeting the possible viral sanctuaries and chronic inflammation. Therefore, an important challenge in understanding the pathogenesis of CHIKV infection was the elucidation of specific host and viral responses in acute and long-lasting conditions to distinguish patterns between patients who either “recovered” or suffered from “chronic” arthralgia 12 mo after the initial insult.

Materials and Methods

Patients and clinical follow-up of the chronic cohort

Patients with clinically acute CHIKVD leading to chronic polyarthralgia were prospectively selected from a large cohort of hospitalized patients (French clinical research hospital program, 2006–2010) during the 2006–2007 outbreaks in La Réunion. These patients were selected to address specifically the mechanisms involved in the long-term physiopathology of CHIKVD and particularly in chronic settings. Informed consent was obtained from CHIKV patients and healthy volunteers from the local medical center (Centre Hospitalier Regional [CHR] Nord Félix-Guyon, St Denis, and local hospital, St. Paul, Ile de la Réunion). All of the biological assays, in particular the levels of proinflammatory cytokines, were also performed in nine patients (so-called positive inflammatory controls) with either CMV or bacterial infections and eight healthy controls (Supplemental Table I). The healthy controls were obtained from the hospital staffs and enrolled with consent. The study was performed with the permission of the coordinating ethics committee of University of Tours (Comité Consultatif de Protection des Personnes se prétenant à des Recherches Biomédicales, Tours and Bordeaux, France). All of the patients and controls were screened by RT-PCR for E1 viral RNA or for the presence of anti-CHIKV IgM/IgG Abs as tested by ELISA (see data listed in Supplemental Table I). The protocol, the sensitivity, and the specificity of these assays have already been reported (29). Briefly, RT-PCR detection of the viral genome was performed after extraction from 200 μl serum either manually using the Viral QIAamp RNA Mini Kit (Qiagen, Courtaboeuf, France) or the MagNa Pure automated system (Roche Diagnostics, Meylan, France) using the High Pure Viral RNA kit (Roche Diagnostics). RT-PCR was performed with the SuperScript One-Step RT-PCR with Platinum Taq kit (Invitrogen, Cergy Pontoise, France). Results are expressed on the threshold cycle values. Ct > 41 cycles was considered as negative for the presence of CHIKV RNA.

The detection of anti-CHIKV IgM was performed by an immunocapture method (Mac-ELISA) according to the techniques and reagents of the National Reference Center of Arboviruses in Lyon, France, as well as the Institut de Médecine Tropicale du Service de Santé des Armées (IMTSSA) in Marseille, France. Briefly, serum IgM was captured by an anti-human IgM (Sigma-Aldrich, I-2386), followed by the addition of CHIKV Ag and detected by hyperimmum murine ascites. Finally, the visualization was performed with an anti-mouse Ab conjugated to peroxidase. (Sigma-Aldrich, A-0168) (16, 29). In our hands, the IgM and IgG OD values of healthy subjects screened as negative controls were <0.01.

Clinical examinations and biological evaluations of the patients were carried out from the first days of clinical acute infection (T0, day of the first visit, i.e., day of referral) and associated with high fever, rash, and arthralgia. Follow-ups were performed at day (D) 15, week (W) 6, and month (M) 3, M6, and M12 PI. The sex ratio of the CHIKVD cohort was 0.63 with 30 women for 19 men, and the mean age was 60.3 y (range 19–90). No
obvious premorbidity medical history was reported apart from a high incidence of metabolic diabetes in 34.7% of the CHIKVD cohort. Patient 21 (male, 59 y old) suffering from chronic arthralgia due to CHIKV relapses and requesting surgery to remove his hygroma was also tested M18 PI. We used the connective tissue biopsies from the surgery to ascertain the presence of the virus and the host inflammatory response in situ. All of the donors provided plasma and serum, and PBMC samples were prepared and stored at −80 and −150 °C, respectively, before use. Levels of several biochemical and cellular surrogate markers were tested. The included markers of cytopenia (neutrophil, platelet, and lymphocyte cell counts) and liver, muscle, and kidney tissue damage (acute phase response, CRP and complement). The inflammatory status was established by immunohistochemistry, ELISA of serum samples, and RT-PCR analyses of PBMCs as described below.

Flow cytometry and immunophenotyping of PBMCs

FACS analysis was limited to only a few patients subsequently found to belong to the chronic group (n = 8) and only one patient from the recovered group. Hence, statistical analyses of the FACS data could not be performed. Typically, PBMCs were isolated by Ficoll-Histopaque (Sigma Diagnostics, St. Quentin Fallavier, France) density gradient centrifugation from whole blood collected in Vacutainers containing acid citrate dextrose anticoagulants. Cells were characterized by immunofluorescence staining. A total of 100 μl whole blood was mixed with a combination of two or three Abs directed against cell surface markers and fixed with R-Phycoerythrin-Texas Red (ECD), R-PE, or FITC. Twelve combinations of Abs were used as follows: CD45-ECD (Beckman Coulter, Villepinte France, A07784)/CD14-PE (Beckman Coulter, A07764)/HLA-DR-ECD (Beckman Coulter, IM3636)/CD80-PE (Beckman Coulter, IM3640), CD86-PE (Beckman Coulter, IM3636)/CD86-FITC (Beckman Coulter, A07751)/CD62L-PE (Beckman Coulter, IM1832)/CD57-FITC (Beckman Coulter, A07170). Goat anti-mouse Alexa Fluor 488 and goat anti-rabbit Alexa Fluor 594 were from Invitrogen Molecular Probes (Cergy Pontoise, France). Rhodamine- or FITC-conjugated goat anti-rabbit and donkey anti-mouse were from Jackson ImmunoResearch Laboratories (West Grove, PA). FITC-conjugated goat anti-human IgG Fab-specific was from Sigma-Aldrich.

Western blot analysis of CHIKV infection and apoptosis

Cells were harvested with a scraper and resuspended in lysis buffer (1 X PBS, 1% Triton X-100, and 1 mM EDTA) containing a protease inhibitor mixture (5 mg/ml PMSF, 100 μg/ml pepstatin A, 100 μg/ml leupeptin, and 100 μg/ml aprotinin; Sigma-Aldrich). Approximately 50 μg of each sample was loaded onto a 4–12% NuPAGE gel from Invitrogen. Membranes were incubated overnight with the different Abs: anti-CHIKV (FDO), anti-cleaved PARP (Promega), anti-CD4 (clone MEM59), anti-CD8 (clone MEM146), anti-CD3 (clone MEM05), and anti-CD141 (BD Biosciences, 559780), respectively. All of the MEM clones were kindly provided by Prof. Vaclav Horejsi (Institute of Molecular Genetics, Prague, Czech Republic).

RT-PCR and qRT-PCR

Total RNA was extracted directly from various tissues, cells, or cell culture supernatant using TRizol reagent (Invitrogen). For RT-PCR, a one-step RT was performed in a final volume of 20 μl containing 0.5 μg extracted total RNA per reaction, 10 μl of 2 X Thermoscript Reaction Mix buffer (Invitrogen), 10 μM of each primer (see Table 1 for primers list), 8 μl of RNase inhibitor (RNasin, Promega), 0.5 μM uracil-DNA-glycosylase (lot no. 12958220, Roche Diagnostics, Meylan, France), and 0.8 μl Thermoscript Plus/Platinum Taq Enzyme Mix (Invitrogen). RT-PCR was carried out in Mastercycler Gradient (Eppendorf Hamburg, Germany) with the following steps: reverse transcription at 50°C for 30 min, amplification at 94°C for 40 s, annealing at 56°C for 30 s and extension at 72°C for 30 s. After 35 cycles, 2.5 μl of the PCR product was analyzed by 1.5% agarose gel electrophoresis and visualized with ethidium bromide. We used the ImageQuant TL software, version 2005, to calculate the size and to semiquantify the amount of each PCR product. The PCR bands were expressed compared with the GAPDH signal.

For qRT-PCR, a 2 X SYBR green PCR master mix (Applied Biosystems, Courtaboeuf, France) was used. The following conditions were used: 95°C for 20 s, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 1 min. Melting curves were generated and analyzed using the Sequence Detection System Software to ensure that the PCR products were specific.

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control for each gene was calculated with the ΔΔCt method using the RT² Profiler PCR Array Data Analysis Template, version 3.0 (SuperArray Bioscience Corporation). Briefly, ΔΔCt = ΔCt(patient) − ΔCt(healthy control) with ΔCt = average Ct − average of housekeeping genes’ Ct (GAPDH, actin). The fold-change for each gene between the patient and the control is calculated as 2^ΔΔCt.

ELISA and Ab array

Chemokine and cytokine serum levels were detected by ELISA using IL-1β (BMS224/2), IL-2 (BMS221/2), IL-4 (BMS225/3), IL-6 (BMS213/2), IL-8 (BMS204), IL-10 (BMS215/2), IL-12 (BMS261/2), IL-13 (BMS231/2), IFN-α (BMS216), IFN-γ (BMS226), and TNF-α (BMS223/3) ELISA module sets (Bender MedSystems, TEBU Bio, France). When only small volumes of biological samples were available (e.g., synovial fluid), we used the Ab array technology to measure the levels of cytokines, growth factors, and chemokines according to the manufacturer’s protocol (RayBio Human Cytokine Ab Membrane Array III, TEBU Bio, France). Fifty microliters of serum or synovial fluid was incubated with the membrane spotted with the different Abs followed by the secondary Ab mix, and the revelation was carried out by ECL (GE Healthcare, Saclay-Orsay, France).

Statistical analysis

All of the results are expressed as mean ± SD and as percentages. The quantitative variables are not normally distributed. As a consequence, the nonparametric tests using exact calculation rather than asymptotic ones have been applied by using StatXact software, version 6 (Cytel Studio, Cambridge, MA). Without any certainty regarding the hypothetical direction of the relation, all of the tests have been interpreted based on a two-tailed p value. The α risk has been chosen at 5%. The Bonferroni correction has been taken into account according to the number of tests done.

Regarding the quantitative variables, the comparison between different groups has been tested by using the Mann-Whitney U exact test. The Fischer’s exact test has been applied to the categorical variables. All p values ≤0.05 were considered statistically significant.

Results

Clinical features of CHIKVD patients leading to prominent chronic arthralgia

Of the 49 hospitalized CHIKVD patients with viral, immunological, and clinical histories, 32 (65%) were tested from the initial period of infection (ranging from 4 to 5 d of symptoms and considered as T0) and up to M12–M18 PI (Supplemental Table I). The other patients were either not willing to contribute to follow-up studies or they were hospitalized but were not enrolled in the cohort study during the acute phase of the disease. The acute infection was always associated with fever (38.1 ± 1°C). The diagnosis was validated either from the RT-PCR data (detection of E1 RNA) (Table I) and/or by ELISA to screen for specific IgM and IgG anti-CHIKV Abs. Nineteen out of 32 hospitalized patients tested at T0 were positive for CHIKV by RT-PCR. No positive RT-PCR result was found at D15, clearly indicating that the virus was rapidly cleared from the systemic blood circulation. We performed standard virus isolation techniques on Vero cells and found that all of the RT-PCR–positive samples yielded a viral clinical isolate capable of developing cytopathic activities (Supplemental Fig. 1). Interestingly, we found that CHIKV-infected Vero cells were...
rapidly dying by apoptosis (cleavage of PARP and detection of apoptotic blebs) (Supplemental Fig. 1).

The chronic status of the disease (i.e., pain with relapsing arthralgia in more than one small articulation 3 mo at least after initial insult) was evaluated using a declarative numerical rating scale as described (20). This scale could be scored between 0 and 10, with higher values indicating more severe pain/arthralgia, and data were recorded at M12 (Supplemental Table I).

Hence, two groups of CHIKVD patients with a complete set of clinical immunological and biochemical data from T0 right to M12–M18 PI were further analyzed comprehensively to identify markers possibly associated with chronic arthralgia post-CHIKV. Group I (5 females/1 male) consisted of CHIKV patients who fully recovered from the acute infection, and group II (8 females/1 male) were patients who experienced chronic relapsing arthralgia at M12 (Table II). The joint pain symptoms reported at M12 (Supplemental Table I) occurred in a fluctuating timely manner on a monthly basis but did not change anatomical location (data not shown). Remarkably, age, viral load, and CRP level at T0 were the three key discriminative factors between the two groups. The chronic status of the disease (i.e., pain with relapsing arthralgia) was more likely to be associated with high viral load at time of referral to the clinic and in elderly patients over 60 y of age (Table II).

However, standard laboratory findings including blood cell count (neutrophil, platelets, and lymphocytes) as well as erythrocyte sedimentation rate, alanine aminotransferase, aspartate aminotransferase, creatine phosphokinase, creatinine, γ-glutamyl transpeptidase, pancreatic lipase, and alkaline phosphatase (ALP) were within normal limits for both groups from T0 up to M12 PI (Table II and data not shown). A mild lymphopenia at T0 was remarkable, but did not change anatomical location (data not shown). Referral to the clinic and in elderly patients over 60 y of age (Table II). The joint pain symptoms reported at M12 (Supplemental Table I) occurred in a fluctuating timely manner on a monthly basis but did not change anatomical location (data not shown). A mild lymphopenia at T0 was more likely to be associated with high viral load at time of referral to the clinic and in elderly patients over 60 y of age (Table II).

We screened for the expression of several inflammatory molecules by ELISA, semiquantitative RT-PCR, and qRT-PCR during the course of CHIKV. Surprisingly, but perhaps in agreement with the mild acute phase response aforementioned, we found that CHIKVD patients (n = 32) had extremely low levels of circulating proinflammatory Th1 cytokines, such as TNF-α and IL-8, during the first days PI (T0). The percentage of CHIKVD patients with positive values above background (detected from healthy controls) is included in Fig. 1. We also screened for IL-1β and IL-2, but they were not detected in CHIKVD patients (data not shown). Anti-inflammatory Th2 cytokines, such as IL-4 and IL-10, were also expressed at low levels. When we compared the recovered (n = 9) versus the chronic (n = 6) group, the overall inflammatory response was more robust in the latter, but this was not statistically different at T0 (Table III). The IFN-γ response was particularly prominent in both groups at T0. IL-12 is essential to initiate the activation of NK cells and macrophages to ward off infectious challenge, and

### Table II. Clinical data (at T0) of CHIKV patients either recovered or suffering from chronic arthralgia M12 PI

<table>
<thead>
<tr>
<th></th>
<th>Virus (per ml Blood) (T0)</th>
<th>Age</th>
<th>Sex</th>
<th>CRP [0–10] (mg/l)</th>
<th>Fibrinogen [2.5–4.5] (g/l)</th>
<th>Platelets [150–450] (10⁹/l)</th>
<th>Lymphocytes [1.2–4.0] (10⁹/l)</th>
<th>Neutrophils [1.4–7] (10⁹/l)</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R1</td>
<td>–</td>
<td>46</td>
<td>M</td>
<td>0.00E+00</td>
<td>78.7</td>
<td>4.2</td>
<td>183.6</td>
<td>0.95</td>
</tr>
<tr>
<td>R2</td>
<td>–</td>
<td>71</td>
<td>F</td>
<td>1.00E+07</td>
<td>47.3</td>
<td>6.2</td>
<td>118.8</td>
<td>1.23</td>
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<td>37</td>
<td>F</td>
<td>0.00E+00</td>
<td>98.4</td>
<td>6.2</td>
<td>32.0</td>
<td>2.21</td>
</tr>
<tr>
<td>R4</td>
<td>+</td>
<td>62</td>
<td>F</td>
<td>0.00E+00</td>
<td>38.4</td>
<td>6.2</td>
<td>32.0</td>
<td>2.01</td>
</tr>
<tr>
<td>R5</td>
<td>+</td>
<td>49</td>
<td>F</td>
<td>4.00E+06</td>
<td>38.4</td>
<td>6.2</td>
<td>32.0</td>
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</tr>
<tr>
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<td>–</td>
<td>37</td>
<td>F</td>
<td>0.00E+00</td>
<td>38.4</td>
<td>6.2</td>
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<td>2.01</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
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<td>50.3</td>
<td>11.33</td>
<td>4.06</td>
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<td></td>
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<td>10.05</td>
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<tr>
<td>Chronic (M12)</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>C1</td>
<td>–</td>
<td>74</td>
<td>F</td>
<td>3.70E+09</td>
<td>10.3</td>
<td>3.8</td>
<td>161</td>
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<tr>
<td>C2</td>
<td>+</td>
<td>90</td>
<td>F</td>
<td>3.00E+09</td>
<td>14.3</td>
<td>3.8</td>
<td>132</td>
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</tr>
<tr>
<td>C3</td>
<td>+</td>
<td>62</td>
<td>M</td>
<td>2.10E+09</td>
<td>188.8</td>
<td>4.6</td>
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<td>C4</td>
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<td>4</td>
<td>124</td>
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<td>+</td>
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<td>F</td>
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<td>46.4</td>
<td>6.4</td>
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<td>F</td>
<td>0.00E+00</td>
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<td>4.4</td>
<td>322</td>
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<td>F</td>
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<td>8.8</td>
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<tr>
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<td>F</td>
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<td>3.9</td>
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<td>70.7</td>
<td>60.2</td>
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<td>183.6</td>
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<td>59.7</td>
<td>0.9</td>
<td>83.4</td>
<td>0.7</td>
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Clinical data at T0 (day of referral) of CHIKV patients classified at M12 PI either recovered or suffering from chronic arthralgia. Diabetes type II status, age (y), sex, CHIKV viral load (qRT-PCR for the E1 gene as expressed as number of RNA copies per milliliter of blood), cell counts, and biochemical analyses are indicated and including physiological range values. The status of chronic arthralgia or recovered was recorded at M12 PI.
interestingly, its level remained dramatically elevated in chronic CHIKVD patients (1650.88 ± 1385.79 pg/ml at M12) (Fig. 1C). In sharp contrast, IL-12 returned to background levels from D15 in the recovered group (Fig. 1C).

The aforementioned data would suggest that circulating immune cells remain activated in patients suffering from chronic arthralgia as recently reported in one patient with post-CHIKV arthritis (20). We were wondering whether this could be further tested by RT-PCR analyses of the patients’ PBMCs comparing samples at T0 and 6–12 mo PI. Due to the limited resources of frozen samples at T0, this study was performed only on patients subsequently categorized as chronic and compared with healthy controls. We performed gene profiling experiments, and the data are presented in Fig. 2. In the first set of experiments, analyses were performed on four chronic patients, and we compared the cytokine and chemokine (see below) profiles at T0 and M6 PI. In essence, the RT-PCR data confirmed high expression of cytokines in CHIKV chronic patient PBMCs at T0 and M6. Strikingly, IL-8, IL-1β, and IL-12 were at least equally or more highly expressed at M6 compared with T0 (with the exception of chronic patient 2). No mRNA expression for IL-4, IL-13, IL-6, or IL-1α was detected, whereas the expression of immunoregulatory cytokines, such as IL-10 and TGF-β1, was demonstrated at T0 and M6 (Fig. 2).

Alphavirus infections, including CHIKV, rapidly induce IFN-α/β production (31, 32). Remarkably, we found high mRNA levels of IFN-α in all four chronic CHIKVD patients, and this expression remained largely elevated in patients at M6 PI (Fig. 2B).
A robust cell-mediated immune response is engaged in CHIKV patients

Having characterized in depth the molecular immune response, we sought to investigate the cellular immune response in CHIKV patients. We wanted to know whether a plausible viral persistence in chronic patients could be associated with poor CD4/CD8 T cell, NK cell, or both responses during the initial acute phase responses. When the data were analyzed retrospectively, only one set of immunophenotyping was performed in 2007 for the recovered group and was compared with nine chronic samples and to 10 healthy CHIKV negative controls. NK cells probably contributed to one of the first lines of defense against the viral infection given that at first evaluation (T0) 66.3 ± 11.1% of the CD3+CD56+ NK cells displayed CD69 in T0 CHIKV chronic patients (e.g., in patient chronic 1, Fig. 4) and therefore were massively activated when compared with healthy controls (9.8 ± 2%) (p < 0.05) (Supplemental Table III). In addition, NK cells were rather spared from the profound lymphopenia with 15.2 ± 4.1% of the CHIKV lymphocytes presenting an NK cell phenotype compared with 8.3 ± 2.5% in healthy controls (p < 0.05). In contrast with NK cells, circulating dendritic cells were detected within normal ranges and did not display an activated phenotype (CD80) (Fig. 4, Supplemental Table III). A relative amplification of plasmacytoid dendritic cells (LinCD11c−HLA-DR+) was demonstrated in the acute phase of the disease (Fig. 4). T cells were also present in normal ranges without dysbalance between TCR-αβ and TCR-γδ subsets (Supplemental Table III). A significant increase in activated T cells was observed in CHIKV patients. We found that 45.6 ± 11.4% of CD3+ T cells expressed CD69 at T0 when compared with 6 ± 1% in healthy controls (p < 0.05). Both CD4+ and CD8+ T cells were involved in such activation with 14.5 ± 3% HLA-DR+CD4+ and 51.7 ± 8.1% HLA-DR+ CD8+ T cells compared with 4.4 ± 0.8% and 12.7 ± 2.7%, respectively, in controls (p < 0.05). The mobilization of memory/effector B cells (CD19+CD27+ IgD−) was particularly prominent in CHIKV patients (47.4 ± 11.6%) when compared with that in controls (18.7 ± 5%) (p < 0.05). Of note, those levels of activation of all T, B, dendritic, and NK cell subsets rapidly returns to normal at 3–6 mo post-CHIKV (depicted only for patient chronic 1, Fig. 4). All in all, our data highlight the massive activation of all of the components of the cell-mediated immune response, with a chief implication of innate NK cells in the acute antiviral response to CHIKV accompanied by a classical mobilization of the adaptive immune T and B cells. Nine chronic CHIKV patients versus one recovered CHIKV patient were analyzed by FACS, and no profound differences were seen apart from a more robust CD4/CD8 activation status in the former group (>50% of CD8 were strongly HLA-DR+ in chronic versus 15.8% in the recovered sample at T0; for the CD4 T cells, <6% were activated in the recovered compared with >14% in chronic). NK cells were equally activated between the two groups (>60% expressed CD69). Statistical analysis could not be performed, and samples from the more recent 2009 CHIKV cases may help to delineate the differences in immune cell activation (34).

CHIKV (mRNA and proteins) persisting in synovial macrophages could contribute to tissue injuries, apoptosis, fibrosis, and an polarized inflammatory response reminiscent of RA

Several lines of evidence from the literature and from data presented herein would argue that alphaviruses can persist and be at the routes of chronic pathologies, such as arthritis evolving to RA (2, 20, 35–37). But where and how does CHIKV hide from the rather robust innate and adaptive immune responses developed by all patients?

To begin to address this question, we performed consented histological assessments of synovial biopsies obtained after volunteered surgery of CHIKV patients. One biopsy was collected during the so-called CHIKV relapses not necessarily associated with fever but nevertheless with severe chronic arthralgia (index 8 on a scale of 10, patient 21). Two more biopsies were obtained from patients hospitalized for orthopedic surgery (not as a consequence of CHIKV). These two patients were positive for anti-CHIKV IgG but did not suffer from chronic arthralgia. All of the analyses were consented for but were limited to surgical cases and were not unnecessarily requested from the other patients of the cohort for ethical reasons. The data are presented in Figs. 5 and 6. Synovial liquid and tissues were collected from surgery (patient 21, inflamed hygroma M18 PI) and analyzed for surrogate markers of CHIKV infection and inflammation. In the synovial fluid, we found that 50% of the infiltrating cells were CD14+ and <5% were...
stained for CHIKV using two different mAbs (Fig. 5Ac). We also found that synovial CD56⁺ NK cells were activated as judged by the CD69 staining (Figs. 5, 6). Circulating NK cells (from PBMCs) from this patient were largely quiescent (CD69⁻CD56⁺ cells) (Fig. 5B). Synovial T cells were mildly activated as judged by HLA-DR staining (CD4⁺CD8⁻) (Fig. 6). The presence of the so-called “ballooned macrophages with multiple vacuoles” as described in RRV (Fig. 5A, May Grunwald Giemsa stain) was concomitant with the detection of high levels of MCP1/ CCL2, IL-6, and IL-8 in the synovial fluid compared with the serum of the same patient (Fig. 5F). These data argue for active monococyte/macrophage trafficking into the synovial tissue of a patient with chronic CHIKVD. Surprisingly, levels of other canonical inflammatory markers known to be elevated in synovial fluid of RA patients were barely affected (i.e., ENA-78, G-CSF, GM-CSF, GRO, GRO-α, I-309, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-7, IL-10, IL-12, IL-13, IL-15, IFN-γ, MCP2, MCP3, M-CSF, MDC, MIG, MIP1β, RANTES, SCF, SDF1, TARC, TGF-β1, TNF-α, TNF-β, EGF, IGF, angiogenin, oncostatin M, thrombopoietin, VEGF, PDGF-BB, and leptin) (38) (Fig. 5F, Ab array detailed in Fig. 6). These data were confirmed at the mRNA levels by RT-PCR (Fig. 5D).

**FIGURE 2.** Proinflammatory cytokines and IFN-α expression in PBMCs from CHIKVD chronic patients at T0 and M6. Semiquantitative RT-PCR was carried out, and data are depicted for four chronic patients. A. Representative data of one agarose gel (n = 3). B. Semiquantitative analyses using the National Institutes of Health imaging software for the data when compared with GAPDH gene expression. C. RT-PCR data of IFN-α mRNA expression in four individual PBMCs from the recovered or the chronic group at M12.
There were several detectable histological abnormalities in the synovium of the patient, including synovial lining hyperplasia (evocative of mild polyarthritis) (Fig. 6C, hematoxylin, eosin, and safran stain), vascular proliferation (CD31/PECAM-1 staining; Fig. 5C), and CD18+ macrophage infiltration remaining mainly at the perivascular level. CD43+ lymphocytes were also present, and subsequent immunostaining indicated that they were mainly CD4+ or CD56+ cells. Cytotoxic CD8+ cells were extremely rare, whereas no CD19+ B lymphocyte cells were detected (Fig. 6). In contrast, quiescent as well as activated NK cells (NKp46 and NKp44, respectively) were distributed in clusters throughout the tissue section (Fig. 6).

Remarkably, the immunostaining for CHIKV using two different mAbs unambiguously stained the perivascular macrophages (Fig. 5C) and identified as CD18+ perivascular cells by double immunofluorescence. Interestingly, high levels of apoptosis, as indicated by immunostaining for cleaved PARP in fibroblasts, was demonstrated in the same tissue section areas (Fig. 6). The presence in situ of CHIKV was confirmed by RT-PCR (and sequencing of the product) for E1 and Nsp2 gene expression (Fig. 5E). Interestingly, the IFN-α signature (mRNA) was concomitant with the persistence of the virus in the synovial tissue (Fig. 6D). In addition to massive apoptosis contributing to the injury of the connective synovial tissue, we found elevated levels of MMP2 in the synovial tissue of chronic patient 21 that may further contribute injuries, lesions, and chronic fibrosis. Full immunohisto logical examination was also conducted in two more biopsies from CHIKVD recovered patients (no relapsing arthralgia) but did not reveal the presence of CHIKV and tissue injuries (absence of cleaved PARP staining; data not shown).

**Discussion**

There is a strong body of evidence that persistent joint manifestations are linked with Barmah Forest virus, Mayaro virus, RRV, O’nyong-nyong virus, and Sindbis virus infections, the so-called arthritogenic alphaviruses but by mechanisms largely ill-characterized (3, 4, 23, 37). The exceptional intensity of the epidemics occurring in a European territory with good medical and university staffs and facilities provided a unique opportunity to describe with modern tools the different aspects of the innate and adaptive immunity generated during the acute phase of CHIKVD.

Moreover, our unique observations that CHIKVD also frequently involves a chronic incapacitating arthralgia/arthritis that can persist for several months raise a number of critical questions, such as the reactivation of virus production, the evasion of the immune antiviral response, the role of an uncontrolled inflammation, and perhaps mechanisms, such as cross-reactivity with self-antigens, a deregulation of autoimmune mechanisms as recently alluded, or both (20, 27). These aspects are of general and great interest in persistent infections and draw attention to the consequences of virus sanctuaries driving long-term illnesses as reported for several RNA viruses (39). Virus sanctuaries in certain tissues not only complicate virus elimination but also enable reactivation during immune suppression (for example, after organ transplantation) (40).

Several stages of the disease can be drawn from our investigations, taking into account the clinical and biological data from patients in acute conditions and several months PI and correlating these findings with the in vitro cell modeling studies.

First, our data highlight a complex interplay between CHIKV and host response in the acute phase of the disease, particularly in elderly patients with high viral burden (>10^9–10^{10} viral particles per milliliter of blood). It has already been established from clinical cohort studies that age is a critical factor linked to more severe CHIKV pathologies and long-term sequelae (11, 19, 20, 41). Of critical note, severe cases reported from hospitalized cohorts may be related to diverse underlying medical conditions, most commonly with hypertension, respiratory conditions, and diabetes mellitus (11, 12, 20, 41, 42). Our study was also from a cohort of hospitalized patients, but we compared two groups where diabetes type II (the only reported comorbidity) was equally found. Remarkably, the patients who will go on to experience chronic arthralgia at M3–M18 PI were over 60 y old and with extremely high viral load at the time of referral to the hospital when compared with the group who will recover from the acute infection with no
detectable or much lower viral load at T0. It is increasingly evident that the elderly are particularly susceptible to severe disease from viral infection, possibly through a dysregulation of their immune system (43, 44), which may be failing to control viral replication.

However, we found that robust cellular and molecular innate immune responses were taking place in all of the CHIKVD patients of both groups as witnessed by the rapid activation of NK cells, plasmacytoid dendritic cells, and T cell subsets at T0. Over 50% of circulating NK and T (CD4 and CD8) cells from the chronic group were activated as assessed by the CD69 and HLA-DR immunophenotyping data, respectively. Counterintuitively, although only one sample could be analyzed, we found that a less pronounced T cell activation was taking place from the recovered patient at T0. Strong immune cell activation to drive the initial antiviral response could

**FIGURE 4.** Rapid and robust mobilization of innate and adaptive immune cells in the acute phase of the CHIKV infection. A, The percentage and the level of activation of T (CD4+ and CD8+) and B lymphocytes (CD19+), dendritic cells (Lin+HLA-DR+) and NK cells (CD3−CD56+) was characterized by FACS analyses of PBMCs from one CHIKV chronic patient (number 23) at T0 and M6 PI when compared with a healthy control. HLA-DR, CD69, and CD23 were used to ascertain for the level of cell activation. Quadrants were established using irrelevant isotype control Abs. B, Cumulative data from eight CHIKVD patients (T0) and eight healthy controls. Significant differences are indicated as (p < 0.05) or as NS. The mean value of the healthy controls is indicated by the gray line.
FIGURE 5. Chronic arthralgia months after acute infection is associated with the persistence of CHIKV in synovial macrophages and accompanied by a polarized inflammatory response. Synovial biopsies from cells (A, B), fluid (F), and tissue sections (C–E) were tested for the presence of CHIKV, inflammation, and immune cell activation several months PI (chronic patient 21, M18). A, Synovial cells including numerous ballooned macrophages (May Grunwald Giemsa stain) were stained for CD14 (red) and CHIKV (green, monoclonal 4F). B, FACS analysis of synovial cells and blood PBMCs from patient 21 at M18. C, Immunoperoxidase brown staining of patient 21 synovial tissue using irrelevant mAb (control), monoclonal anti-CHIKV, monoclonal anti-CD18 (macrophages), monoclonal anti-CD43 (lymphocytes), and monoclonal anti-CD31 (endothelial cells). D and E, The expression of inflammatory markers and CHIKV RNAs (E1 and Nsp2) was tested by RT-PCR of synovial tissue. F, Ab array technology (RayBio) was used to screen for the level of cytokines and chemokines in synovial fluid and control serum sample from patient 21. MCP1, IL-6, and IL-8 were particularly highly expressed in synovial fluid, whereas the levels of ENA-78, G-CSF, GM-CSF, GRO, GRO-α, I-309, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-7, IL-10, IL-12, IL-13, IL-15, IFN-γ, MCP2, MCP3, M-CSF, MDC, MIG, MIP1β, RANTES, SCF, SDF1, TARC, TGF-β1, TNF-α, TNF-β, EGF, IGF, angiotensin, oncostatin M, thrombopoietin, VEGF, PDGF-BB, and leptin were not affected.
lead to the exhaustion of T cells, and this has been linked subsequently to viral persistence (45, 46). Although this phenomenon is well-described for several RNA viruses, it remains to be studied for arboviruses. It will be important to test the effector functions of the T cells, but ongoing ELISPOT analyses in our laboratory clearly indicate that CD4 and CD8 cells are highly capable of recognizing several CHIKV Ags and producing high levels of IFN-γ (data not shown). The rapid clearance of the CHIKV in patients' sera at D15 is a clear testimony to the competent antiviral cellular and humoral responses at least from a systemic point of view.

The Th1 cytokine response is essential to promote immune cell activation, but they can also contribute to tissue injuries as, for example, in RA. We were however surprised to note that proinflammatory TNF-α, IL-6, and IL-8 were poorly expressed in CHIKD sera and in sharp contrast to other viral and bacterial infections tested concomitantly (47, 48). Several studies have reported the regulation of the cytokine response in a cell culture system exposed to alphaviruses, but little was known in patients (for review, see Ref. 3). Moreover, we found that IL-4 and IL-13 (Th2 cytokines) were expressed in very few CHIKD patients, whereas low expression of IL-10 was demonstrated by RT-PCR but not by ELISA. There are some discrepancies between the cytokine ELISA data from sera and the RT-PCR data of PBMCs. Soluble cytokines can be rapidly degraded in serum, and the qRT-PCR data were

**FIGURE 6.** Polarized cellular inflammatory response in chronic form of CHIKVD. A, List of inflammatory markers screened using an Ab array. Samples from chronic patient 21 (C11, M18 PI) was analyzed by FACS, Ab array, and immunohistochemistry. B, Immunophenotyping of PBMCs of patient 21 (C11) from blood and synovial fluid. C, Synovial tissue (hygroma) of chronic patient 21 was stained for hematoxylin, eosin, and safran, CD4, CD8, NKRp44, and apoptosis (cleaved PARP). a, Original magnification ×100; b–f, original magnification ×600.
obtained solely from PBMCs, excluding the likely contribution of other cell types (endothelial cells, fibroblasts, and hepatocytes) to the systemic inflammatory response. IL-12 is known to act synergistically to promote innate immune cell activation (NK cells and macrophages to thwart off an infectious challenge), and it was highly elevated in the majority of CHIKV patients at T0. More importantly, its expression remained elevated throughout the time course of CHIKV in chronic patients, and this would argue for the hypothesis of a persisting infectious challenge months after the initial insult. This hypothesis was further substantiated by the detection of high levels of IFN-α mRNA only in chronic but not recovered CHIKV patients at M6 and M12.

CHIKV may trigger persistent joint pain and arthritis-like pathology with mechanisms possibly involving not only host-derived inflammatory cytokines but also the virus itself hijacking the “soldier” of the innate immune system, the resident tissue macrophages as described for RRV (3). We found that NK cells infiltrating the synovial tissue in close vicinity to chronically CHIKV-infected macrophages displayed an activated phenotype (NKp44-positive) (49). In contrast, the lack of infiltrating CD8+ cells in the synovial tissue is surprising and may contribute to the persistence of CHIKV. The expression of antiviral type I IFNs was demonstrated in the PBMCs of chronic CHIKV patients and in the synovial tissue with latent CHIKV infection. In the CHIKV+ biopsies (synovial tissue and fluid), we found gene expression of IFN-α and IL-10 but not of proinflammatory cytokines (TNF-α, IL-1β, and IFN-γ). Chemokines, such as CCL2 found in the synovial tissue, are also likely to be involved in the chronic phase response to promote leukocyte trafficking through activated endothelial cells. These findings are in agreement with a canonical chronic immune response reminiscent of but distinct from RA. The absence of polymorphonuclear cells in the synovial fluid and the paucity of proinflammatory cytokines, such as TNF-α and IL-1β, are important components to consider while selecting the best treatment regimen for chronic CHIKV.

It is plausible that CHIKV persisting in immunoprivileged niches (called here sanctuaries) contributes directly to synovial tissue damage. RRV was shown to persist in synovial macrophages, and it is interesting that this behavior can be extrapolated to CHIKV (for review, see Ref. 2) and, in general, to many RNA viruses. There is an emerging paradigm that chronic infection of macrophages by some viruses reprograms their differentiation toward a so-called “alternative phenotype” different from the M1/M2 characteristics and contributing to tissue fibrosis (50–53). It will be interesting to ascertain whether this is also true for CHIKV. We found that CHIKV has profound cytotoxic effects and can induce apoptosis in vitro and in synovial tissues as assessed by the presence of numerous cleaved PARP-positive cells, whereas high expression of MMP2 may contribute to chronic tissue lesions. It remains to be tested whether phagocytosis of apoptotic cells (containing or not remnants of CHIKV) by the neighboring macrophages may permit viral persistence in a nonphlogistic environment and whether novel strategies could be identified to curtail viral persistence in the macrophage reservoir (54).

All in all, the fundamental and clinical questions addressed in acute and chronic CHIKV are applicable to many RNA viruses causing pathologies with the possibility to link clinical observations with experimental data. The vast body of recent information on the molecular biology, cell biology, and pathogenesis of CHIKV and other alphaviruses makes this system a powerful tool for fundamental and clinical research and to shed new light on the possible routes to protective vaccines and treatments (55). Patients with post-CHIKV RA-like arthritides are currently and efficiently treated with methotrexate (10–20 mg/wk for months), but this drug has also immunosuppressive activities that may complicate the issue of possible viral reactivation (20, 27).

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Disclosures

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References


