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Active Infection of Human Blood Monocytes by Chikungunya Virus Triggers an Innate Immune Response

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Chikungunya virus (CHIKV) is an alphavirus that causes chronic and incapacitating arthralgia in humans. To date, interactions between the immune system and the different stages of the virus life cycle remain poorly defined. We demonstrated for the first time that CHIKV Ags could be detected in vivo in the monocytes of acutely infected patients. Using in vitro experimental systems, whole blood and purified monocytes, we confirmed that monocytes could be infected and virus growth could be sustained. CHIKV interactions with monocytes, and with other blood leukocytes, induced a robust and rapid innate immune response with the production of specific chemokines and cytokines. In particular, high levels of IFN-α were produced rapidly after CHIKV incubation with monocytes. The identification of monocytes during the early phase of CHIKV infection in vivo is significant as infected monocyte/macrophage cells have been detected in the synovial tissues of chronically CHIKV-infected patients, and these cells may behave as the vehicles for virus dissemination. This may explain the persistence of joint symptoms despite the short duration of viremia. Our results provide a better understanding on the basic mechanisms of infection and early antiviral immune responses and will help in the development of future effective control strategies. ***The Journal of Immunology*****, 2010, 184: 5903–5913.**

Chikungunya virus (CHIKV) is the causative agent for Chikungunya fever (CHIKF) was first described in 1952 during an epidemic in Tanzania, East Africa (1, 2). It is a positive-strand RNA virus belonging to the *Togaviridae* family and *Alphavirus* genus, and is maintained in two distinct transmission cycles; (1) sylvatic cycle and (2) human–mosquito–human cycle. The scale of epidemics for the former is smaller and is mainly confined within Africa involving primates such as monkeys and forest-dwelling *Aedes* mosquitoes. CHIKV is mainly transmitted by *Aedes aegypti* and *Aedes albopictus*. Since then, CHIKF epidemics have often been characterized by long interepidemic periods of >10 y (3) in many parts of Southern and Southeast Asia (4–7). During the last 8 y, major outbreaks have occurred among islands in the Indian Ocean (3), with Reunion Island being one of the most severely hit, with one-third of its population infected, and >240 deaths (8). During the same period in 2006, the virus also entrenched itself in India and caused an outbreak of unprecedented magnitude, affecting >1.39 million people (9), with a total of 2944 deaths reported during the epidemic (10). Outbreaks then spread to several countries in Southeast Asia, including Singapore (11, 12). Infection by CHIKV is usually nonfatal and self-limiting. Clinical features are characterized by symptoms such as fever, headache, rash, and arthralgia, which may last for days, whereas in some cases, chronic arthritis may persist for years. Atypical clinical complications such as acute nephritis, severe acute hepatitis, and meningoencephalitis have also been reported from recent outbreaks (13–16).

CHIKV infection in humans is thought to begin with the inoculation of viruses after a bite by CHIKV-infected *Aedes* in the dermis of the host. From there, the virus will find its way into the blood vessels before dissemination to the target tissues/or organs (17). Although the exact route and mechanisms of early infection is poorly defined (18), previous studies from other alphaviruses have indicated the involvement of different immune cell populations in the skin (19–22), and migrating cells such as macrophages and/or dendritic cells (DCs) (19). The events taking place during the acute blood phase of CHIKV infections have not been clearly defined. Although the viremic period in humans is relatively short (23, 24), the plasmatic levels of virus can go up to very high levels (3.3×10^9 viral copies/ml) in CHIKV-infected patients (24). Such high levels of circulating virus suggest that blood leukocytes could be infected by CHIKV and involved in viral production. However, previous work reported that blood leukocytes were not susceptible to CHIKV infection in vitro, suggesting that the blood virus were produced by cells from other tissues (22). This was surprising because other arboviruses have been shown to target diverse blood cells in vivo and in vitro such as monocytes, DCs, or B cells (25, 26). Deciphering if blood leukocytes are targets for CHIKV infection is important because many blood leukocyte subsets, and in particular monocytes, are involved in innate immune responses against viruses, and in the

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The online version of the article contains supplemental material.

Abbreviations used in this paper: CHIKF, chikungunya fever; CHIKV, chikungunya virus; CHIKV-HI, heat-inactivated CHIKV; DC, dendritic cell; HEK, human embryonic kidney cell; hpi, hours postinfection; mDC, myeloid DC; MFI, mean fluorescence intensity; MOI, multiplicity of infection; pDC, plasmacytoid DC.

control of viral infections. These early responses play a role in shaping subsequent antiviral adaptive immune responses and may influence the development of immune-pathogenesis.

In this study, we investigated whether blood leukocytes were infected in vivo during natural CHIKV infections in humans using clinical specimens collected from the second and third CHIKF outbreaks in Singapore (12). We showed that blood monocytes are the main targets for CHIKV during the acute phase of infection. These observations were further substantiated in vitro using whole blood or primary monocytes obtained from healthy donors. Interactions of the virus with monocytes, and to a lower extent with B cells and myeloid DCs (mDCs) in vitro induce a rapid innate immune response, mainly characterized by the production of antiviral cytokines such as IFN-α. Our study identified blood monocytes as an important target for early CHIKV blood infection and may explain CHIKV pathogenesis because macrophages have been shown to act as reservoirs in persistent viral infections in the macaques model (27) and in chronic patients (J.J. Hoarau and P. Gasque, unpublished data).

Materials and Methods

Patients

Patients were recruited from the infectious diseases clinics and wards of the National University Hospital, a 900-bed teaching hospital that serves the western sector of Singapore. The patients' ages ranged from 19 to 64 y (median, 40 y) (Supplemental Tables I and II). The majority (85%) had no comorbidities, whereas two patients had hypertension and ischemic heart disease. Blood specimens were obtained with informed consent (the study was reviewed and approved by the institutional review board at the National Healthcare Group with Domain Specific Review Board no. DSRB E/08/414). The number of days from onset of illness to sampling ranged from 1 to 43 d (median, day 6). All cases had complained of fever with median duration of 3 d (range 1–10 d). Other common symptoms included myalgias (85%), arthralgia (78%), rash (78%), gastrointestinal symptoms of diarrhea, vomiting or abdominal pain (35%), and headache (44%). Two patients required hospitalization, and the remaining were treated as outpatients. Clinical features associated with severe CHIKF such as arthritis, neurologic involvement, and hemorrhagic manifestation were not observed. Laboratory findings showed mean nadir lymphopenia and mild thrombocytopenia of $0.79 \pm 0.50 \times 10^9$/l and $168 \pm 100 \times 10^9$/l, respectively, occurred on median 4 d after illness onset.

Primary cells isolation

A total of 15–30 ml blood were collected in EDTA tubes from CHIKV-infected patients and healthy donors, respectively. PBMCs were isolated from total blood by gradient centrifugation using Ficoll-Hypaque. Monocytes from healthy donors' PBMCs were positively selected using anti-human CD14 microbeads according to manufacturer's instructions (Miltenyi Biotec, Auburn, CA). Purity was >95% as verified by flow cytometry.

Cells

African green monkey kidney epithelial cells (Vero-E6) and human embryonic kidney cells (HEK 293T) were cultured in DMEM, supplemented with 10% FBS. Primary cells, including monocytes were maintained in IMDM (Hyclone, South Logan, UT) supplemented with 10% heat-inactivated human AB serum (Innovative Research of America, Novi, MI). All media and reagents were tested negative for endotoxins.

Virus stocks

CHIKV isolates used in this study were originally isolated from a French patient returning from Reunion Island during the 2006 CHIKF outbreak (28) and from Singapore in 2008 at the National University Hospital. Virus stocks were prepared via numerous passages in Vero-E6 cultures, titered, washed, and precleared by centrifugation before storing at −80°C. All virus stocks were titered by plaque assay and quantified by quantitative RT-PCR.

In vitro virus infections

CHIKV infections on whole blood (from healthy donors) were performed using multiplicity of infection (MOI) 1 and 10 (to total number of leukocytes). Each infection mix consisted of 1 ml citrate blood and 1 ml virus suspension prepared in serum-free IMDM. Samples were incubated at 37°C for 24 h with intermittent shaking. Infections using heat-inactivated CHIKV (CHIKV-HI) were performed in a similar manner, whereas mock infections using the cell-free fractions from Vero-E6 cultures were performed in parallel.

Total number of monocytes isolated from each healthy donor varied between 30×10^6 and 50×10^6 cells. Monocytes were inoculated with CHIKV suspension with a MOI of 50 in 15 ml Falcon tubes in serum-free IMDM, and incubated at 37°C for 1.5 h with intermittent shaking. Mock infections were performed in parallel as described previously. Monocyte suspensions were then precleared by centrifugation at 1500 rpm for 5 min, and resuspended in IMDM containing 10% heat-inactivated human AB serum. The monocyte suspensions were then seeded equally into 60-mm diameter tissue culture dishes and incubated at 37°C before harvesting at different hours postinfection (hpi).

Secondary virus infections were performed on HEK 293T monolayers with 1 ml collected cell-free supernatant from CHIKV-infected monocytes cultures (collected at 24 hpi) in 60-mm diameter tissue culture dishes. Cells were incubated at 37°C as described previously and were harvested at 24 hpi.

Cell harvest

Cell-free supernatants were collected and precleared at 1500 rpm for 5 min for cytokine measurements, and also for secondary viral infections on HEK 293T monolayers. Monocyte cell pellets were resuspended in 1 ml ice-cold 1× PBS (without Ca^{2+} and Mg^{2+}, pH 7.3), and 500 μl were used for surface markers characterization, whereas the other 500 μl were used for intracellular flow cytometry analyses.

Flow cytometry

Detection of CHIKV Ag in HEK 293T cells, monocytes, and CHIKV-infected patients' PBMCs were carried out in a two-step indirect intracellular staining process. An additional surface staining step was performed on patients' PBMCs to discriminate between the different cell types present in PBMCs. Abs were used to identify $CD4^+$ Th, $CD56^+$ NK cells, $CD3^+$ T cells, and $CD8^+$ T cytotoxic, $CD303^+$ plasmacytoid DCs (pDCs,), $CD1c^+$ mDCs, $CD14^+$ monocytes, and $CD19^+$ B cells. CD45 Ab was also included for the identification of pan-leukocytes. Except for Abs against CD56, CD1c, and CD303, which were purchased from Miltenyi Biotech, other Abs were from BD Biosciences (San Jose, CA) . For patients' PBMCs, thawed PBMCs were fixed with 1 ml 1× FACS lysing solution and permeabilized in 1 ml 1× FACS permeabilization solution 2. To minimize nonspecificities, 20 μl FcR blocking reagent (Miltenyi Biotec) per 10^7 PBMCs was added and incubated at room temperature for 10 min. PBMCs were stained with a mAb recognizing a CHIKV Ag. The mAb recognizes an Ag expressed only postinfection. This Ab does not detect E1 and E2 gp, and capsid protein. It presumably recognizes a non-structural protein. Further characterization will be described elsewhere (unpublished data). The Ab (10 μg/ml) was incubated for 30 min, followed by APC-conjugated goat anti-mouse IgG $F(ab')_2$ Ab (10 μg/ml, Invitrogen, Carlsbad, CA) for 30 min. Cells were then washed twice with 1× ice-cold PBS before staining with the cell specific Abs described. Data was acquired in BD LSR II (BD Bisosciences) using BD FACSDiva software. Depending on availability, 15,000–100,000 cells were acquired. Dead cells and duplets were excluded in all analyses with FSC/SSC gating. Results were analyzed with FlowJo version 7.5 β software. Detailed descriptions could be found in the supplemental methods. For HEK 293T and primary monocytes, cell pellets were first fixed with 1× FACS Lysing Solution (BD Biosciences), followed by permeabilization with 1× FACS Permeabilization Solution 2 (BD Biosciences) for 10 min at room temperature on 96-wells round bottom tissue culture plates. Cells were first stained with the mouse Ab recognizing CHIKV for 30 min, followed by Alexa-Fluor 488-conjugated goat anti-mouse IgG $F(ab')_2$ Ab (10 μg/ml, Invitrogen) for 30 min.

Surface molecules characterization on monocytes was carried out at each time point using PE conjugated Abs (10 μg/ml) against HLA-DR, CD54, CD106, CD31, CD86, and CD14 (BD Biosciences) for 15 min in 100 μl volumes. FcR blocking reagent was used to minimize nonspecific binding. Data was acquired in BD FACSCalibur (BD Biosciences) and BD FACS CellQuest Pro software, and analyzed with FlowJo version 7.5 β software. Results were normalized with PE conjugated isotype control IgG2a and IgG1 Abs (BD Biosciences), and are presented as mean fluorescence intensity (MFI).

Viral RNA extraction and viral load analysis

Viral RNA was extracted from patients' blood and CHIKV-infected cell cultures using QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Quantification of the extracted

RNA was determined using a TaqMan assay modified from Pastorino et al. (29). Viral load was estimated from a standard curve generated using serial dilutions of synthetic CHIKV E1 RNA transcripts. The limit of detection was 10^2 RNA copies/ml.

Virus plaque assay

Vero-E6 cells were seeded at 2.5×10^5 cells per well in 24-wells plates and incubated at 37°C overnight. Cell culture media were gently aspirated from the wells and the cells were washed once with 1× PBS. Ten-fold serial dilutions of virus mixture were prepared in Hank's Buffer (Sigma-Aldrich, St. Louis, MO). Then, 0.1 ml virus mixture was inoculated into each well and incubated for 1 h at 37°C. After 1 h of adsorption, virus overlay were removed and washed once with 1× PBS. One milliliter of 1% carboxymethylcellulose (w/v) (Sigma-Aldrich) in DMEM with 5% FBS was layered onto the infected monolayers. The plates were incubated in a humidified incubator at 37°C with 5% CO_2 for 3 d. Virus plaques were visualized by staining the monolayer with 1 ml 0.5% crystal violet/10% formaldehyde solution (Sigma-Aldrich) for 2 h at room temperature and virus plaques were counted after thorough washing with water.

Multiplex microbead immunoassay

Levels of cytokines and chemokines from cell-free supernatants were measured using the Biosource Human Cytokine Assay kit (Invitrogen) as described previously (30). Values below the limit of detection for each factor were considered negative.

Results

Detection of CHIKV in monocytes during acute viremic phase of CHIKF patients

Fourteen patients with clinical acute disease were recruited during the second and third CHIKF outbreaks in Singapore (12). Clinical examinations and laboratory evaluations of these patients were carried out according to standardized protocol. All patients had high fever, rash, and arthralgia (*Materials and Methods*, Supplemental Tables I, II). It was noted that all the 14 CHIKV-infected patients presented marked leucopenia during the acute phase of the infection in agreement with previous studies (23, 30). As a result of this phenomenon, reduced amounts of viable PBMCs were obtained and processed from all the patients.

To determine whether PBMCs were infected by CHIKV during acute in vivo infection, we first verify whether CHIKV RNA could be detected in those cells. Although limited in quantity, viral RNA was obtained from a representative patient "CHIKV1," and was successfully amplified and quantified (Supplemental Fig. 1*A*). This demonstrated that the PBMCs were infected by CHIKV and the virus particles were present inside the PBMCs.

To determine which PBMC subset(s) might harbor the virus during acute in vivo CHIKV infection, flow cytometry analysis was performed using a mAb that detects a CHIKV Ag, which is expressed only intracellularly postinfection (Supplemental Fig. 1*B*) together with different sets of Abs against NK cells ($CD3^-CD56^+$), $CD3^+$ T cells (both $CD4^+$ and $CD8^+$), monocytes ($CD14^+$), B lymphocytes ($CD19^+$), mDCs ($CD19^-CD14^-CD1c^+$), and pDCs ($CD19^-CD14^-CD303^+$).

As shown in Fig. 1*A*, flow cytometry analysis of the different PBMC subsets of the representative patient (CHIKV1) with high viremia at the acute phase (1 d after disease onset), not only revealed a pronounced decreased in the overall number of PBMCs (Fig. 1*A*), but also in the percentage of mDCs and B lymphocytes when compared with those from healthy controls (Fig. 1*A*). These alterations in the PBMCs were transient as the cell numbers of the different populations returned to similar levels as those of the healthy controls 66 d after disease onset when the leukocytes' profile of the same representative patient was analyzed (Fig. 1*B*). CHIKV Ag was detected in monocytes, and to a lower extent in pDCs from the same patient (Fig. 1*A*, *lower panel*). CHIKV Ag was not detected in NK cells, $CD4^+$ and $CD8^+$ T cells (Supplemental Fig. 2). The very low number of mDCs in PBMCs precluded the meaningful assessment of CHIKV Ag detection in this cell population. CHIKV Ag detection was specific because staining was not observed in monocytes or in other cell subsets from noninfected healthy controls (Fig. 1*A*, 1*C*). In PBMCs isolated from the same CHIKV1 patient 66 d after the onset of disease, when CHIKV RNA was no longer detectable in the blood by RT-PCR, it was observed that CHIKV Ags could not be detected in both the monocytes and other PBMCs subsets (Fig. 1*B*, *lower panel*).

When PBMCs from the other 13 CHIKV-infected patients were analyzed by flow cytometry, some contained a higher percentage of CHIKV Ag positive monocytes than others (Fig. 1*C*). This heterogeneity was found to be associated with the different levels of blood viral load (Fig. 2*A*). Indeed, when the 14 patients were separated into two groups based on differences in viral load (Fig. 2*A*, 2*B*), it was observed that patients with high viral load had higher percentages of CHIKV Ag positive monocytes per total PBMCs or per total monocytes than the low viral load group (Fig. 2*C*). Moreover, there was a clear correlation between CHIKF patients' viral load and percentages of CHIKV Ag positive for monocytes per total PBMCs or per total monocytes (Fig. 2*D*).

In vitro infection of whole blood from healthy donors with CHIKV

Viruses isolated from these 14 patients were all from the East-, Central-, South-African (31) genotypes: A226 or A226V that both have been involved in the recent outbreaks (12). We first compared the in vitro infectivity of a representative virus isolate, the IMT CHIKV strain, previously isolated from a patient from Reunion Island in 2006 (28), and the SGP11 strain (containing the A226V mutation) isolated during the 2008 outbreak in Singapore. Both strains did not have any significant differences in terms of viral infectivity and viral load (Supplemental Fig. 3). So we used the IMT CHIKV strain in subsequent experiments.

We next tested whether CHIKV infection can occur in human whole blood, a system that more closely mimics the in vivo setting. Whole blood contains the different blood cell subsets in normal distribution and allows analysis of early viral innate response of the different immune cells in a more physiological context (32). Whole blood samples from three healthy donors were inoculated with the IMT strain CHIKV at different MOI conditions (MOI 1 and 10), and cells were collected at 24 hpi. Control infections using heat-inactivated virus were performed in parallel for all experiments because CHIKV-HI does not infect and replicate in susceptible cell lines even at high MOI (Supplemental Fig. 4). CHIKV Ags were positively detected in the monocytes and to a lesser extent in B cells and mDCs. The number of CHIKV Ag-positive cells increased in a dose-dependent manner in these subsets (Fig. 3*A*, 3*B*). CHIKV Ags were not detected in monocytes or B cells when incubated with CHIKV-HI by flow cytometry for all the donors (Fig. 3*A*, 3*B*) indicating that detection of CHIKV Ag was not due to passive intake of virus particles but to active virus infection. Some mDCs (~4% of total mDCs) were stained positively for CHIKV Ags with CHIKV-HI (Fig. 3*A*), indicating that these cells may have phagocytosed some CHIKV-HI. Viral load was also measured by quantitative RT-PCR using viral RNA isolated from the cell-free supernatant of whole blood cultures. High levels of CHIKV infection and replication were observed under these conditions for live CHIKV infection (up to 10^9 RNA copies/ml was detected in the three different donors) (Fig. 3*C*).

In vitro infection of primary monocytes with CHIKV

Having demonstrated that monocytes from acute CHIKF patients were positive for CHIKV Ags substantiated by whole blood CHIKV-infected cultures, we next showed the infections of CHIKV in

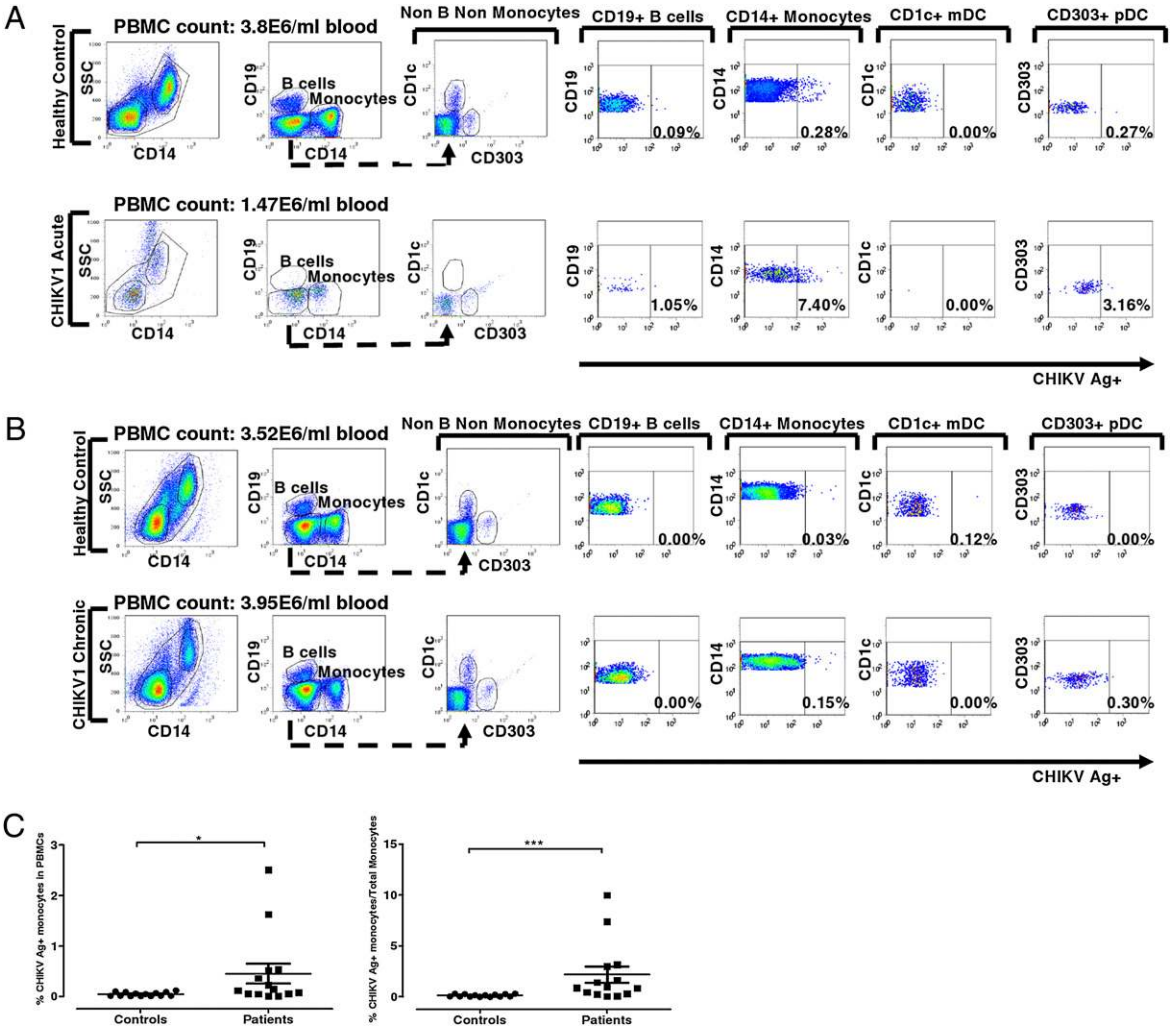

FIGURE 1. Simultaneous detection of CHIKV Ags in immune cell subsets. *A*, PBMCs from healthy control (*top panel*) and CHIKV-infected patient (*bottom panel*) were first stained with mouse mAb recognizing CHIKV expressed only postinfection, followed by goat anti-mouse APC-conjugated Ab. A total of 15,000–100,000 cells were acquired. The different PBMCs subsets were identified with cell-specific Abs as described in *Materials and Methods*. Data shown are from a representative acute high viremic CHIKV-infected patient (CHIKV1) and a healthy control. Percentage of the different cell populations: B cells (4.76%), monocytes (35.65%), mDCs (1.12%), and pDCs (0.72%) for the healthy control; and B cells (2.27%), monocytes (28.36%), mDCs (0.07%), and pDCs (3.76%) for the CHIKV1 patient. Percentage of CHIKV Ag positive (Ag^+) cells are indicated. *B*, Profile of patient CHIKV1 66 d after disease onset, and a healthy control. Percentage of the different cell populations were as follows: B cells (4.62%), monocytes (25.57%), mDCs (0.88%), and pDCs (0.32%) for the healthy control; and B cells (4.68%), monocytes (12.60%), mDCs (0.81%), and pDCs (0.38%) for the CHIKV1 patient. Percentage of CHIKV Ag^+ cells are indicated. *C*, Expression of CHIKV Ag^+ monocytes per total PBMCs (*left panel*) or per total monocytes (*right panel*) in 14 healthy controls and 14 acute CHIKV-infected patients. Data are presented as mean % of CHIKV Ag^+ monocytes ± SEM. $*p < 0.05$ and $***p < 0.001$, Mann-Whitney *U* test.

monocytes because these cells were predominantly infected after 24 h. $CD14^+$ monocytes isolated from PBMCs of four healthy donors were infected in vitro with the IMT strain of CHIKV at MOI 50. Cell infectivity and viral replication in monocytes were assessed by flow cytometry and quantitative RT-PCR at different times postinfection. The number of CHIKV Ag positive monocytes increased over time, and reached a peak of 36.42% ± 16.1% at 12 hpi (Fig. 4*A*, Supplemental Fig. 5). As a control, the nonmonocytes fractions from the different donors' PBMCs were also infected with the same amount of CHIKV. The number of infected cells in this fraction (including the B cells and mDCs) was significantly lower (Fig. 4*A*, Supplemental Fig. 6). This observation confirmed that monocytes were the main targets for CHIKV infection. CHIKV RNA was detected from the in vitro-infected monocytes but the viral RNA load decreased over time (Fig. 4*B*). Depending on the individual donors, viral loads increased and peaked between 6 and 24 hpi indicating viral RNA production. For some donors, no increase in viral RNA load was observed (Supplemental Fig. 7). To determine whether virions were produced, virus plaque assays were performed next, and it was observed that they were produced but production decreased over time (Fig. 4*C*). To assess if the newly released virions were infectious, secondary infections were performed using HEK 293T cells (a highly susceptible HEK cell line) from cell-free supernatant collected at the 24 hpi time point, when viral load was $\sim 10^8$ RNA copies/ml (Fig. 4*B*). Virions obtained from the cell-free supernatant of the CHIKV-infected monocytes cultures from three different donors infected HEK 293T cells with high efficiency (Fig. 4*E*, *top panel*). Moreover, high viral loads were produced after 24 h of infection (up to 10^{12} RNA copies/ml) (Fig. 4*E*, *lower panel*), confirming the infectious nature of the CHIKV virions obtained from the CHIKV-infected monocytes cultures. These experiments revealed that in vitro infection of monocytes by CHIKV is efficient (up to 40% of monocytes), and that virus replication occurs in these cells. However, virus replication is rapidly controlled and virus particles production decrease rapidly over time. During the 48-h culture, we observed that a high cell death

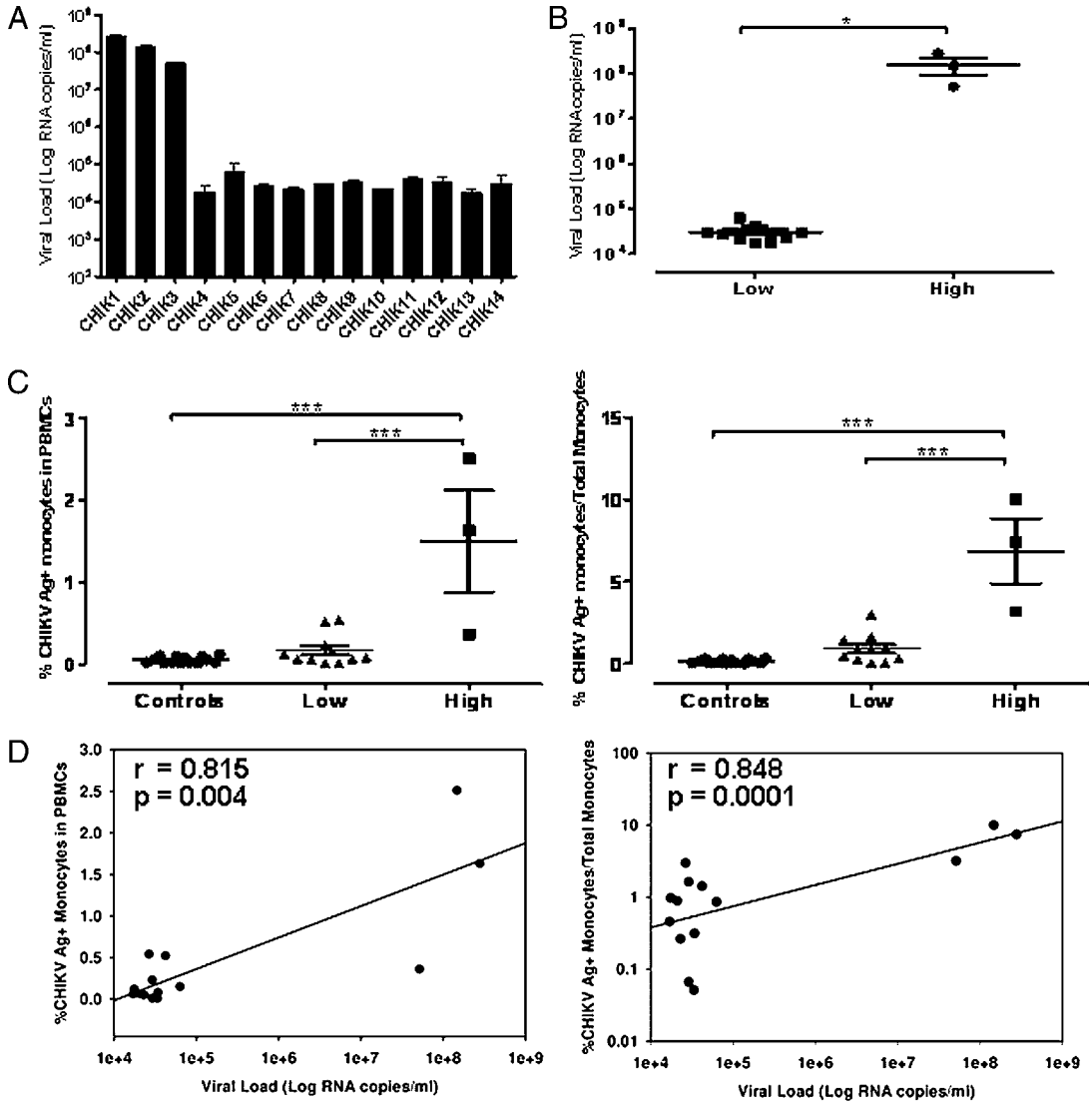

FIGURE 2. High percentage of CHIKV Ag^+ monocytes is associated with high viral load. *A*, Viral RNA was extracted from 14 acute CHIKV-infected patients' blood and viral load were quantified by quantitative RT-PCR. Quantitation was performed in duplicates and results are presented as mean log viral RNA copies/ml ± SEM. Limit of detection was 10^2 RNA copies/ml. *B*, CHIKV-infected patients were further segregated into two groups (low and high viral load). $*p < 0.05$, Mann-Whitney *U* test. *C*, The expression of CHIKV Ag^+ monocytes in total PBMCs (*left panel*) or per total monocytes in 14 acute CHIKV-infected patients (with low and high viral load) and in 14 healthy controls. $***p < 0.001$, ANOVA test, Bonferroni post test. *D*, Linear regression analysis of CHIKF patients' viral load and percentage CHIKV Ag^+ for monocytes per total PBMCs (*left panel*); and per total monocytes (*right panel*) in 14 acute CHIKV-infected patients. The correlation (*r* values) and *p* values between CHIKF patients' viral load and percentages CHIKV Ag^+ for monocytes were indicated.

occurred in our monocytes cultures after CHIKV infection (Fig. 4*D*). This is not surprising as CHIKV is known to be cytopathic (22), similar to other alphaviruses with continual cell death in the in vitro cultures between 24 and 48 hpi (33, 34). However, it is worth mentioning that a large fraction of human monocytes (up to 60%) in the mock-infected cultures also died over the 48-h period in culture conditions devoid of macrophage growth factors (35–37). Thus, the rapidly decreasing numbers of monocytes available for new infections over time explains in part the decrease of CHIKV viral RNA production, and the declining production of virus particles.

Activation profile of monocytes after in vitro infection with CHIKV

To evaluate the impact of CHIKV infection on the phenotype of monocytes, expression levels of CD14, HLA-DR, CD86, and some adhesion molecules on viable monocytes were assessed by flow cytometry at different time points after in vitro infection with CHIKV (Fig. 5, Supplemental Fig. 8). Expression of CD14 was transiently reduced by 2.2 ± 1.4-fold at 24 hpi (MFI: 242.6 ± 95.4 from monocytes in infected cultures containing ~40% of infected cells, [Fig. 4*A*] versus 465.8 ± 81.3 for mock-infected monocytes, $p < 0.05$, Mann-Whitney *U* test). HLA-DR expression (MFI: 807.9 ± 87.8 from monocytes in infected cultures versus 691.4 ± 323.5 for mock-infected monocytes, $p = 0.35$) was not observed up to 48 hpi (Fig. 5*B*). Different adhesion markers such as CD86, CD31 (PECAM-1), CD54 (ICAM-1), or CD106 (VCAM-1) were also tested but their expression did not differ overtime between the two groups (data not shown). This suggested that monocytes were not activated directly by the virus, or nonspecifically by soluble factors such as cytokines released by infected cells.

Cytokine and chemokine secretion profiles of monocytes from naive donors after CHIKV infection

Profiles of 16 monokines and chemokines known to be produced by monocytes were analyzed using a multiplex-microbead immunoassay using cell-free supernatant from monocytes cultures

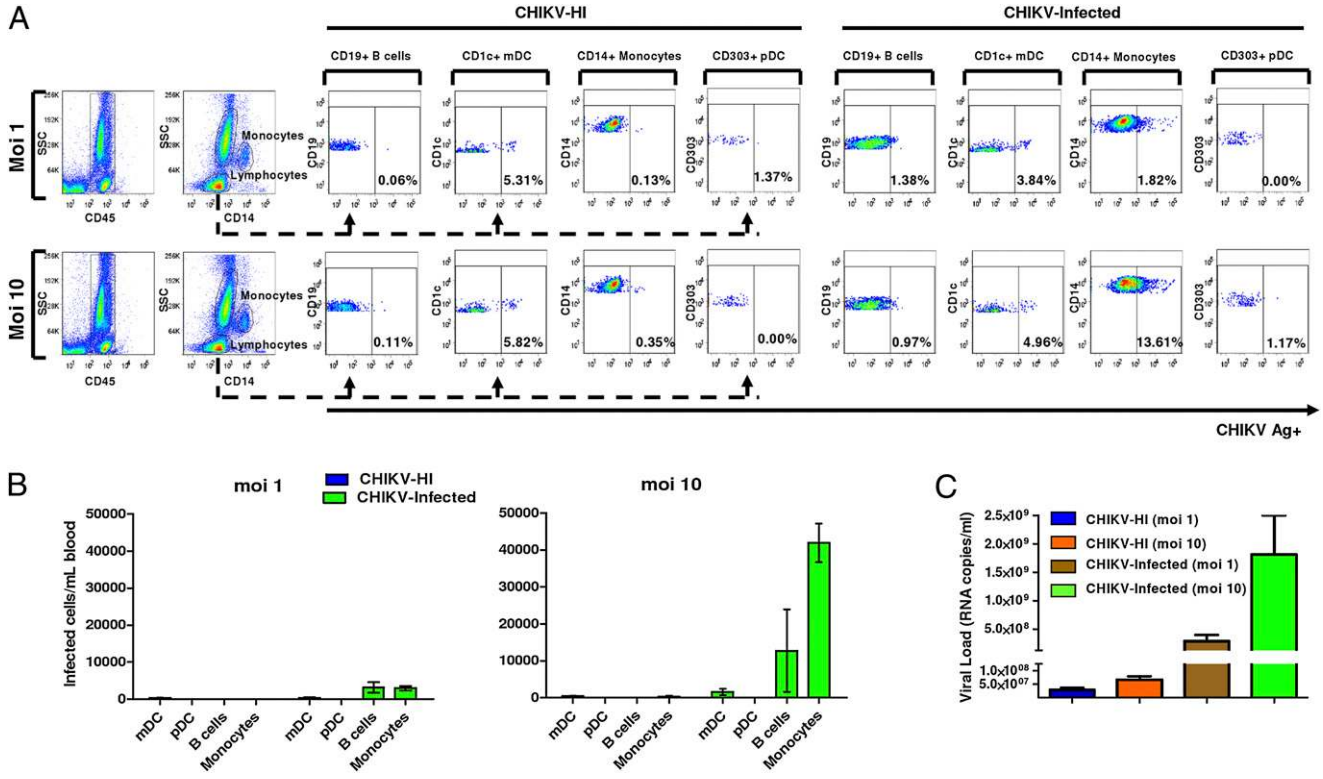

FIGURE 3. CHIKV inoculation in human whole blood. Human whole blood was collected from three healthy donors and infected with CHIKV-HI (heat-inactivated) as a negative control infection or with CHIKV at both MOI 1 and MOI 10 for 24 h. *A*, Percentage of CHIKV Ag^+ $CD14^+$ monocytes was analyzed by flow cytometry as described in *Materials and Methods*. *B*, Number of CHIKV Ag^+ cells are indicated. The number of infected B cells and monocytes were calculated from the percentage CHIKV Ag^+ cells acquired from flow cytometry with respect to the total number of cells obtained for each subset from the BD Trucount TBNK reagent. pDCs and mDCs cell numbers were calculated based on the number of events acquired from flow cytometry with respect to the $CD45^+$ population. The number of infected cells was then calculated accordingly. *C*, CHIKV viral load after CHIKV infection of human whole blood was quantified using quantitative RT-PCR, and data are expressed as mean ± SEM of viral RNA copies/ml. The limit of detection was at 10^2 RNA copies/ml.

collected during the time-course infection as described previously. Only the levels of IL-8, MIP-1β, RANTES, IL-1Ra, IP-10, IFN-α, and IL-12 in CHIKV-infected monocytes were significantly altered postinfection when compared with mock-infected monocytes (Fig. 6, Supplemental Fig. 9). Levels of IL-1Ra, IP-10, IFN-α, and IL-12 were observed to be significantly elevated ($p < 0.05$, Mann-Whitney *U* compared against mock-infected cultures) postinfection. Production of IFN-α and IL-12 was swift because high levels were produced in CHIKV-infected monocytes cultures in <2 h after virus inoculation. In contrast, IL-8, MIP-1β, and RANTES levels were significantly decreased ($p < 0.05$, Mann-Whitney *U* compared against mock-infected cultures). It is interesting to note that although levels of IL-8 and MIP-1β were decreased across the entire time course of infection, RANTES levels recovered and returned to basal levels at 24 hpi. Significant changes were not observed for nine other cytokines and chemokines (IL-1β, IL-6, TNF-α, GM-CSF, monokine induced by IFN-γ, MCP-1, IL-10, MIP-1α, and G-CSF) analyzed (Supplemental Fig. 9). It has to be noted that mock-infected monocytes did release some monokines and chemokines over time possibly due to apoptosis (35, 37, 38) and may in turn further activate the remaining viable monocytes.

Cytokine and chemokine secretion profiles of whole blood from naive donors after CHIKV infection

Experiments on whole blood were performed to evaluate the participation of monocytes in the blood innate response of CHIKV-infection. We stimulated whole blood samples from normal healthy donors with CHIKV at two different MOI conditions. Cell-free supernatant samples were collected at different time points, levels of cytokines and chemokines were measured. CHIKV induced a significant innate immune response as characterized by the higher levels of immune mediators as compared with the levels observed from CHIKV-HI or mock in whole blood samples (Fig. 7). These immune mediators include IL-1Ra, IL-6, IFN-α, and chemokines such as IP-10, MCP-1, and MIP-1β (Fig. 7).

When compared with the responses observed with purified monocytes from healthy donors as described previously (Fig. 6, Supplemental Fig. 10), it was found that IFN-α and IP-10 were increased in both experimental settings suggesting that monocytes are directly responsible for the increased levels of these factors. Interestingly, in whole blood preparation, IL-1Ra, was produced faster than when monocytes were incubated alone with CHIKV. This suggest that other cell subsets may either produce these mediators or provide additional signals (either by cell contact or by soluble factors) to stimulate monocytes to produce these cytokines more rapidly.

Discussion

Disease caused by CHIKV is characterized by an early acute phase that could last from day 2 to day 10 after illness onset (17, 24). During this period, CHIKV viremia increases and acute inflammation occurs, followed by dissemination of the virus to secondary infection sites that may include the brain, liver, spleen, but predominantly the joints (17). For several viral infection models, the host's innate immunity is known to play a key role during this period so as to suppress virus propagation and dissemination before an adaptive immune response is activated. Blood leukocytes

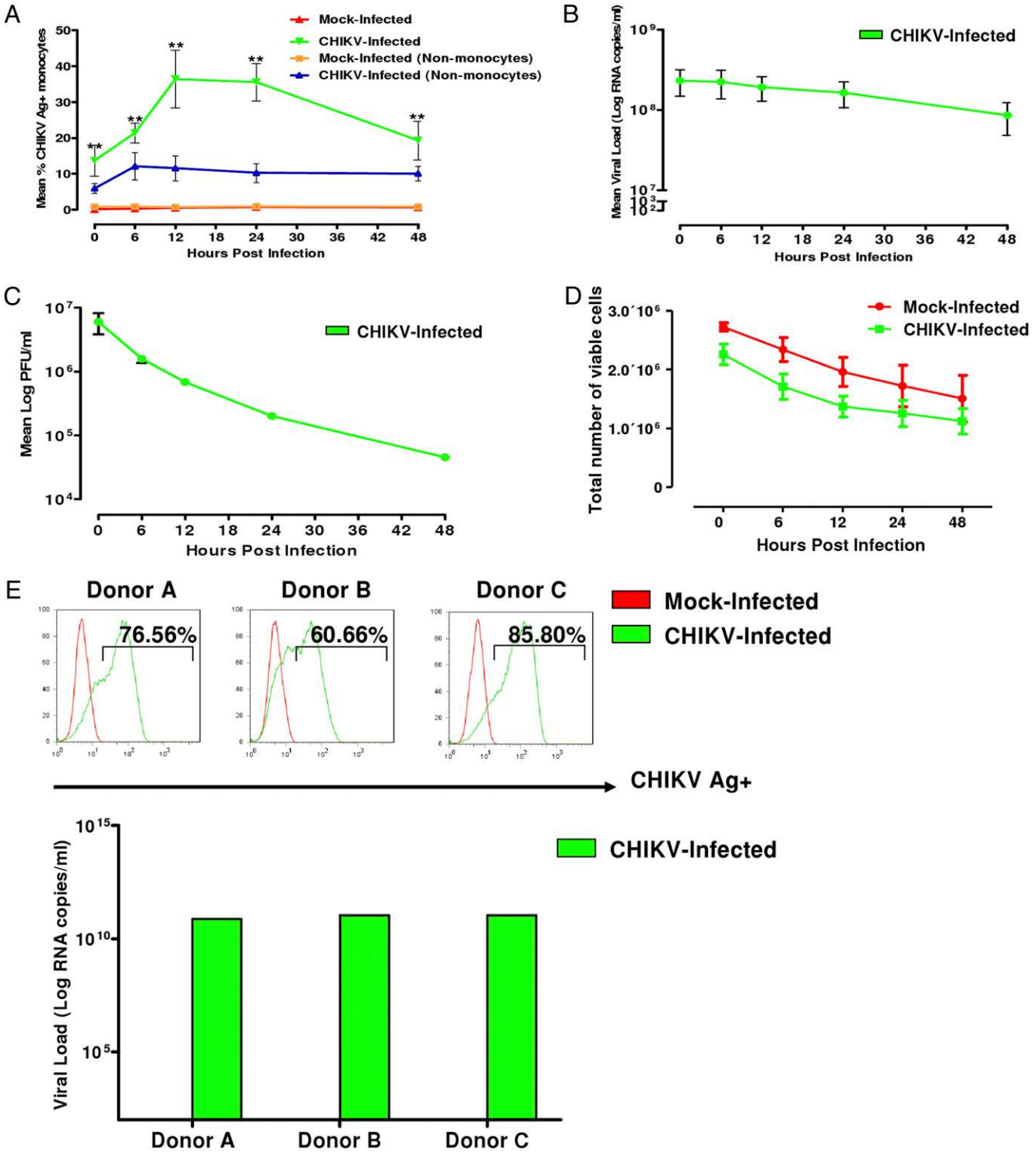

FIGURE 4. Time course analysis of CHIKV infection on isolated monocytes from four healthy blood donors. *A*, Mock-infected and CHIKV-infected monocytes cultures harvested at 0, 6, 12, 24, and 48 hpi was analyzed by flow cytometry. The percentage of CHIKV Ag^+ monocytes was detected at each time point. Data are expressed as mean ± SEM of four different donors. $^{**}p < 0.01$, Mann-Whitney *U* test between CHIKV-infected and mock-infected monocytes. *B*, CHIKV viral load was quantitated using RT-PCR at the different time points indicated. Data are expressed as mean log viral RNA copies/ml ± SEM of four different donors. Limit of detection was 10^2 RNA copies/ml. *C*, Plaque assay expressed as mean log PFU/ml ± SEM of four different donors. *D*, Total number of viable cells were counted from four different donors at different times post infection. *E*, Secondary CHIKV infection of HEK 293T cells from CHIKV-infected monocytes culture supernatant. Cell-free supernatant collected at 24 hpi fractions from mock-infected and CHIKV-infected monocytes cultures were used to infect HEK 293T cells. Cells were harvested at 24 hpi and measured by flow cytometry for percentage of CHIKV Ag^+ cells (*top panel*), and CHIKV viral load (*bottom panel*) was quantified using quantitative RT-PCR. Data are expressed as log viral RNA copies/ml and limit of detection was 10^2 RNA copies/ml.

such as monocytes, DCs, and NK cells are the main components of innate immunity, and they have been implicated in the immunopathogenesis of many viral diseases (25, 39–41). These cells are attractive virus targets because they are located mainly in the circulation and peripheral tissues and can assist virus dissemination (40).

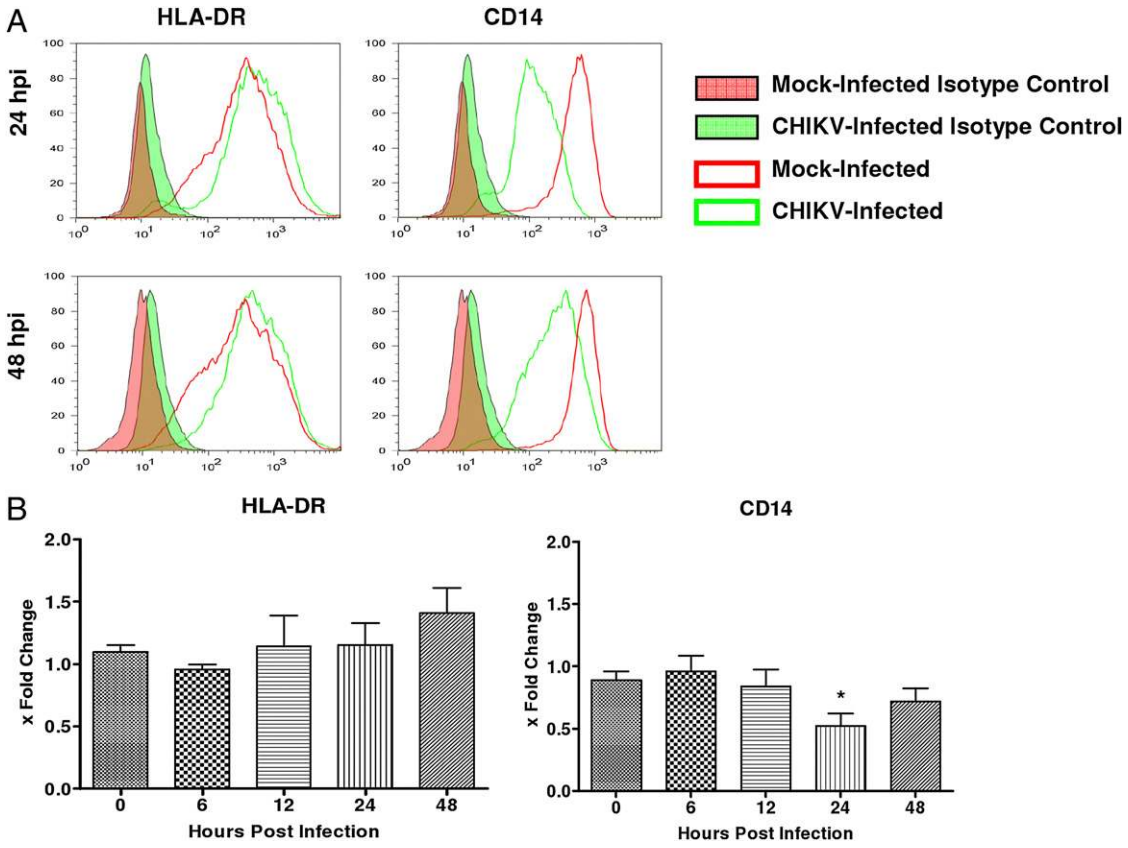

FIGURE 5. Comparison of surface molecules expression on monocytes postinfection with CHIKV. *A*, Flow cytometry analysis of monocytes from a representative healthy donor at 24 and 48 h after in vitro infection with CHIKV. Surface molecule expression on mock-infected and CHIKV-infected monocytes cultured cells were stained with PE-conjugated Ab against HLA-DR and CD14. *B*, Histograms of the expression of HLA-DR and CD14 on monocytes. Shaded histograms indicate isotype controls, whereas unshaded histograms indicate immunostained populations. MFI of mock-infected and CHIKV-infected monocytes was normalized with MFI of isotype controls. Ordinate indicates x-fold change for HLA-DR and CD14 compared with mock-infected monocytes. Results are represented as mean ± SEM of four independent experiments. $*p < 0.05$, Mann-Whitney *U* test between CHIKV-infected and mock-infected monocytes CD14 expression at 24 hpi, respectively.

In this study, we sought to identify the principal target cell(s) of CHIKV in the blood of acutely infected patients and how infection of these cell subsets could induce an antiviral innate immune response. First, using unfractionated PBMCs from CHIKF patients and a flow cytometry approach, we identified blood monocytes as the main targets for CHIKV. Although some positive staining for

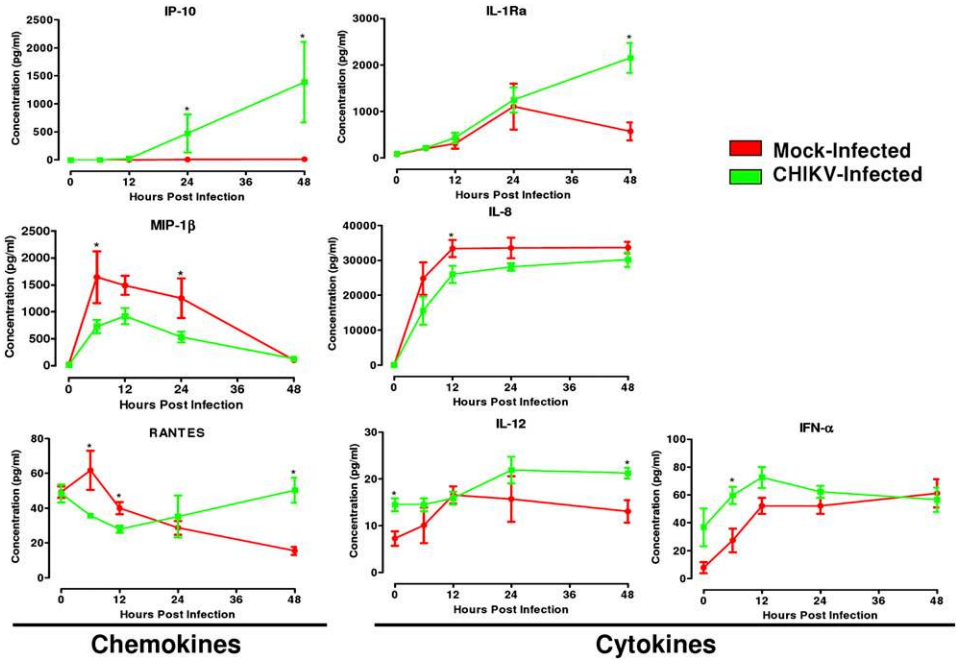

FIGURE 6. Cytokine and chemokine production by monocytes postinfection with CHIKV. Monocytes from four healthy donors were infected in vitro with CHIKV as described in *Materials and Methods*. The levels of cytokines and chemokines from the cell-free supernatant of CHIKV-infected and mock-infected cultures were measured at different time points indicated by Luminex 200 system with the multiplex microbeads immunoassay. Results are expressed as mean ± SEM of four different donors. $*p < 0.05$, Mann-Whitney *U* test.

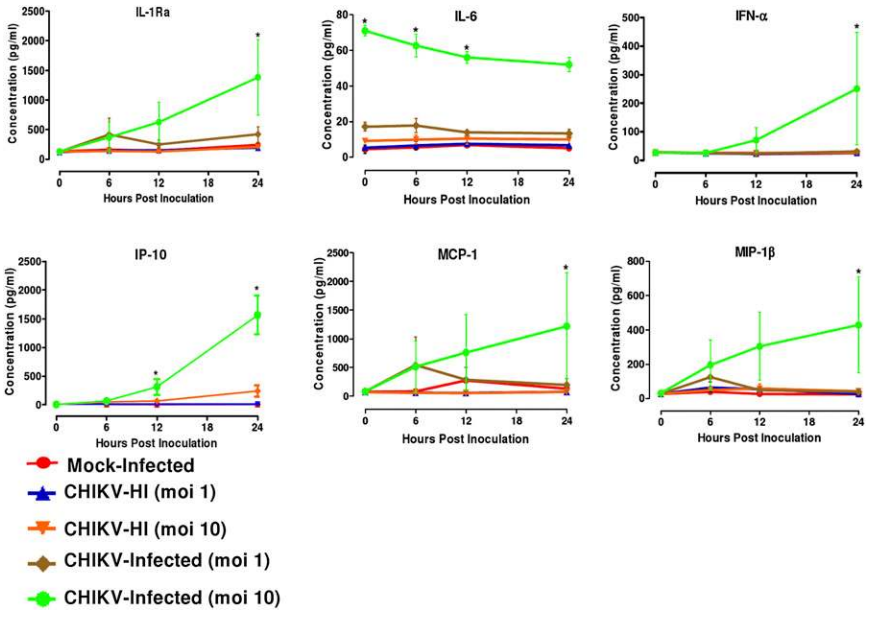

FIGURE 7. Cytokine and chemokine release by human whole blood exposed to CHIKV. Whole blood from three healthy donors were incubated as indicated: Mock-Infected, CHIKV-HI, or with CHIKV (CHIKV-infected) at both MOI 1 and MOI 10. The levels of cytokines and chemokines from cell-free supernatant were measured using the Luminex 200 system. Data are expressed as mean ± SEM of three independent donors. $*p < 0.05$, Mann-Whitney *U* test.

DCs (pDCs) was observed, their very low numbers in the periphery in infected patients precluded drawing any significant conclusions (Fig. 1*A*, 1*B*). However, in whole blood experiments we did observe positive staining for CHIKV Ags in B cells and mDCs, suggesting that these cells were to some extent susceptible to infection. Using a whole blood in vitro infection model, we further demonstrated that CHIKV Ag can be detected predominantly in the monocytes after CHIKV inoculation (Fig. 3). Finally, using purified $CD14^+$ monocytes, we were able to show that these cells were indeed infected with CHIKV (Fig. 4). In addition, CHIKV could replicate in monocytes, but it was not extensive, and in vitro viral production declined quickly over time (Fig. 4). The likely reason for this phenomenon could be due to the strong inhibition of CHIKV replication by IFN-α, a cytokine that was shown to be produced readily in vitro (Fig. 6, and see *Discussion* below) and after CHIKV infection in humans (30). B cells were also infected with the virus as shown in the whole blood model. However, the number of B cells decreased sharply in this system, suggesting a cytopathic effect of the virus. This correlated with findings in patients in whom strong B cell depletion was observed.

To date, testing of the susceptibility of human immune cells to CHIKV infection has only been demonstrated in vitro (22). Contrary to our findings, it was reported that monocytes and other leukocyte subsets were resistant to CHIKV infection. However, macrophages derived from monocyte cultures supplemented with specific growth factors did support viral infection and replication. Likely explanations for the discrepancy could be due to differences in monocyte culture conditions used in the two studies. Indeed, contrary to Sourisseau et al. (22), we used heat-inactivated human AB serum and a higher MOI in our monocytes infection studies. We chose to use human serum to simulate the in vivo situation. However, CHIKV infection did occur in monocytes using medium without human AB serum (data not shown). We tested the importance of different MOI conditions in whole blood experiments and observed that the number of infected monocytes depended on the MOI used. At a MOI comparable to that used by Sourisseau et al. (22), infection was still observed.

These data support the hypothesis that monocytes become a significant target for CHIKV only when high levels of virus are present in the circulation. Indeed, detection of CHIKV Ag in monocytes positively correlated with blood viral load (Fig. 2*C*, 2*D*). Patients with higher viral loads were patients diagnosed early after the onset of the disease, possibly close to the peak of viremia. Because monocytes postinfection do not produce high levels of infectious virus, this suggests that CHIKV may be produced in another compartment. It has been shown for some alphaviruses that early viral production may take place in the lymph nodes (19). More recently, P. Roques and collaborators (27) detected CHIKV replication in the lymph nodes of macaques in the first 2–3 d postinfection. In this model, replication was shown to occur in monocyte/macrophages, which infiltrated the infected lymph nodes. This is in agreement with Sourisseau et al. who demonstrated CHIKV replication in the in vitro cultures of macrophages (22).

We next investigated whether CHIKV interactions with the monocytes might activate these cells. First, flow cytometry analysis of in vitro infected monocyte cultures showed that expression of macrophage activation markers such HLA-DR and adhesion molecules CD54 (ICAM-1), CD106 (VCAM-1), and CD31 (PECAM-1) were not modified after CHIKV infection. Only a transient downregulation of CD14 surface expression was observed. Decrease in CD14 expression on blood monocytes were reported in septicemic patients (42–44) and has been associated with apoptosis of monocytes (45) that was also observed in our culture conditions. This suggested that interaction with CHIKV did not lead to obvious monocytes activation. Moreover, it was recently reported that expression of CD14 was downregulated in the differentiated state in a stimulus-directed manner (46) such as infection. However, CHIKV (but not CHIKV-HI) infection induced a rapid specific cytokine response. Consistently, and in <2 h in cultures containing monocytes alone, high levels of IFN-α were detected (Figs. 6, 7). Because CHIKV is exquisitely sensitive to IFN-α (22, 47), this swift response may explain the rapid control of CHIKV replication in the blood. IL-12, a proinflammatory cytokine, was also produced rapidly by monocytes in <2 h after interactions with CHIKV. Although this cytokine has no direct antiviral role, it is known to activate NK cells to increase antiviral activities (48, 49). IFN-α that was also produced by monocytes alone or in whole blood cultures postinfection at later time points (24–48 h), have also been shown to possess similar activities on NK cells (50). The role of NK cells during CHIKV infection is unknown at this point, and due to their importance in antiviral

activities, further studies will be crucial. Also, the proinflammatory chemokines IP-10, MIP-1β, IL-8, and to a lesser extent RANTES rapidly produced by monocytes in acute CHIKV infection. MCP-1 was only detected in the whole blood cultures, and may be produced by other leukocyte subsets. The roles of these chemokines in CHIKV infection are unknown and deserve more studies in future.

Interestingly, two of the main proinflammatory cytokines, IL-1α/β and TNF-α were not produced in the two in vitro systems. This is surprising because these cytokines have been implicated in the development of joint pathologies, a common feature of CHIKV infection. Recently, these factors have been detected in the blood of patients with chronic disease (L. Ng, unpublished data). This suggests that production of these two factors is a late event, and may be due to cells in the target organs such as the joints. This would explain why arthralgia may persist after the virus has disappeared from the circulation. IL-6 has been previously associated with fever during CHIKV infection (30) but the source of this cytokine has not been identified. In this study, we have shown that IL-6 was rapidly produced in whole blood cultures but not in the monocytes cultures postinfection. This pattern of production suggested that either other cells were involved in the production of this cytokine or that monocytes require additional signals to produce it. IL1-Ra, an IL-1 antagonist, was produced at high levels in monocytes or whole blood cultures after CHIKV infection. Balance between IL-1 and IL1-Ra has been shown to influence the development of many human diseases, in particular, rheumatoid arthritis (51). We did not observe the production of IL-1 in monocytes or in whole blood cell cultures. However, recently, we have shown that IL-1β was detected in the plasma of patients (51). More importantly, IL-1β was shown to be a marker of severity during CHIK infection, suggesting that it was secreted by cells other than blood cells. Thus, increased production of IL-1Ra may help counteract the development and the extent of the proinflammatory response induced by CHIKV.

There is growing evidence that macrophages play an important role in the development of CHIKV-induced pathology. Recent CHIKV infection studies in the macaque model (27) and in humans (J.J. Hoarau and P. Gasque, unpublished data) have found that synovial macrophages were infected by CHIKV. It was also described in the macaque model that mononuclear infiltrations were observed. Our data suggest that infected blood monocytes may behave as the Trojan horse and disseminate the virus to these particular sites. Some of these sites could become opportune sanctuaries supporting persistent viral replication, despite the general immune response. Defining the phenotype of the migrating monocytes is of interest because we have shown in this study that monocytes infected in vitro did not have an increased expression of the common adhesion molecules (ICAM-1, VCAM-1, or PECAM-1). The implication of other adhesion molecules such as those from the VLA family remains to be determined, as well as the pattern of chemokine receptors they express, because these molecules may direct the infected monocytes to specific tissues as demonstrated for rheumatoid arthritis (52).

In conclusion, we have shown that blood monocytes are the main targets of CHIKV during acute phase infection at a time where high levels of virus are detectable in the blood circulation. Although these cells might be infected, they did not produce substantial levels of new viruses. However, infection led to the development of a swift cytokine and chemokine response. Cytokines, such as IFN-α, were rapidly produced and helped to control viral replication. Lastly, infected monocytes might help viral dissemination in organs such as joints and muscles, which form the basis of the debilitating joint disease characterized in CHIKF.

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Disclosures

The authors have no financial conflicts of interest.

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