Effect of Toll-like receptor activation on Chikungunya virus replication, proinflammatory cytokine production, Type I interferons and interferon-stimulated genes expression in monocytes and MDM in-vitro infected

Running title: Chikungunya virus infection of Monocytes and MDM and inflammatory response

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ABSTRACT

Introduction

The chikungunya virus (CHIKV) is an emerging virus that has generated major problems in public health, due to the epidemic's outbreaks in tropical y subtropical regions. The CHIKV is transmitted by the bite of infected *Aedes* mosquitoes, and is known to target different human cells types, such as primary monocytes and macrophages. The CHIKV recognition by pattern recognition receptors, including toll-like receptors, expressed by monocytes and macrophages leads in the inflammatory response, including type I interferon (IFN-I), which is involved in innate immune response. The IFN-I induced the expression of IFN-stimulated genes that protect the cells against viral infection. However, monocytes and macrophages have been linked to severe symptoms, due to the high production of IL-6 and TNF-α, two proinflammatory cytokines. Herein, this study aimed to evaluate if Toll-like receptors activation influence CHIKV replication, proinflammatory cytokine production, IFN-I and IFN-stimulated genes expression in monocytes and monocyte-derived macrophages (MDM) in vitro infected with CHIKV.

Methodology

Monocytes and MDM were infected with CHIKV and co-stimulated with TLRs agonists. The viral titers were performed by plaque assay method in the cells culture supernatants. Furthermore, the culture supernatants were used to quantify the pro-inflammatory cytokines by ELISA and the level mRNA of ISGs were performed by RT-PCR.

Results

We found that both monocytes and MDM are target cells of CHIKV infection, with a maximum peak at 24 h.p.i. and then the production of infectious viral particles drops dramatically. Furthermore, although the activation of TLR-2/6, -3 and -4 do not have a significant effect in CHIKV replication, the activation of TLR-7/8 with R848 agonist significantly decreased the production of infectious viral particles from 6 h.p.i until 48 h.p.i. Likewise, we found a statistically significant increase in the production of IL-6 and TNF-α at 48 h.p.i. Besides, a slight increase in the IFN-beta mRNA level was observed in monocytes at 6 h.p.i., in response to TLR-7/8 activation, as well as a slight increase in the level of the PKR mRNA at 24 h.p.i. and a significant increase in OAS2 at 6, 24 and 48 h.p.i. but it is statistically significant, only at 48 h.p.i. However, in MDMs the activation of TLRs did not

regulate viral replication, although a tendency in decrease TNF-alpha was observed at 6 and 24 hpi with the activation of TLR-4 and a tendency to increase IL-6 and TNF-alpha in response to activation of the TLR-7/8.These results suggests a difference in the activation of the two cell populations to CHIKV infection and shed light on the importance of monocytes and macrophages in the pathogenesis and/or resolution of CHIK fever.

Conclusion

The results suggest that monocytes and MDMs are target of CHIKV infection with a maximum peak at 24 h.p.i. but both inflammatory and antiviral responses are different in the two cell populations.

Key words: , Pattern recognition receptors, Toll-like receptors, monocytes, macrophages, proinflammatory cytokine Antiviral activity, ISG.

Introduction

Chikungunya fever is a disease whose etiologic agent is Chikungunya virus (CHIKV), which is transmitted by the bite of infected *Aedes* (*Ae*) mosquitoes, mainly *Ae. albopictus* and *Ae. aegypti* in tropical and subtropical areas. The febrile illness is characterized for clinical symptoms, such as arthralgia, fever, myalgia, headache among other symptoms. The illness is auto limited, however in some patients the arthralgias can persist for months to years, which involves higher morbidity associated with the infection [1].

CHIKV is an Alphavirus and belongs to the family Togaviridae. It is an enveloped, spherical and icosahedral virus, with 65-70 nm in diameter, and their genome is a single-stranded positive sense RNA (ssRNA) of approximately 11.8 Kb in length. This ssRNA is composed of two open reading frames (ORFs), the one on the 5' end encoded non-structural proteins (nsP1-4) and the second one at the 3' end that encoded five structural proteins: capsid protein (C), envelope glycoproteins (E1 and E2), and two small cleavage products (E3 and 6K) [2],[3],[4].

The virus was initially isolated in Tanzania in 1952 of the serum of patients during an epidemic period. However, it was only since 2005 that CHIKV has garnered international attention due to the outbreaks that happened mainly in India (over 1 million) and Reunion Island approximately 300000 cases with higher severity symptoms [1],[5],[6]. In 2013 CHIKV came to the Americas, with the arrival of imported cases to the Caribbean islands, such as Saint Martin, Dominica y Martinica and later in South America which is an endemic zone for the other arbovirus. Colombia reported about 236.000 cases, being one of the countries in the region with more reports [7],[8].

After CHIKV is inoculated by the bite of the infected female mosquito into the skin, they carry out a primary replication cycle in fibroblasts and mesenchymal cells [9],[10]. Later through lymphatic route reaches its main target organs such as liver, muscle, heart, mainly joints. In murine models have been observed higher infiltration of monocytes and macrophages and several studies suggest that these cell populations are involved in pathogenesis and/or the resolution of CHIKV infection [11].

Monocytes and macrophages express pattern recognition receptors (PRRs), among them, the family of the Toll-like receptors (TLR) that recognize Pathogen-associated molecular patterns (PAMPs) such as dsRNA, ssRNA, proteins, among others, that trigger the

production of proinflammatory cytokines, including IL-6, IL-8, IL-1β, TNF-α that are associated with severity of during the acute phase of the disease. However, also drive transcription of type I Interferon (IFN β, IFN α) and Interferon stimulated genes (ISG) such as protein kinase R (PKR) and Oligoadenylate synthetase (OAS). The product of those genes has been implicated in inhibition of proteins synthesis and degradation of viral RNAs, inducing an antiviral state involved in controlling the infection. Nevertheless, is unknown the role of some PRRs expressed by monocytes and macrophages in the resolution of CHIKV infection [12],[13],[14],[15]. Thus, in the present study we pretend evaluate the modulation of the viral replication, production of proinflammatory cytokines and the expression of antiviral genes in response to the activation of some TLRs in CHIKV infected monocytes and macrophages derived of monocytes (MDM).

Methods and results

Viral Stock

CHIKV was a kind gift from Dr. Francisco Javier Díaz (Grupo Inmunovirología, Facultad de medicina, Universidad de Antioquia) amplified from a Colombian patient's serum sample and propagated in Vero cells. Vero cells were infected with CHIKV, at a 1.0 multiplicity of infection (MOI) and incubated at 37°C and 5% CO_2 for 3 days or until an advanced cytopathic effect was observed. Next, the supernatant was collected, precleared by centrifugation (2000 rpm/10 min) and the viral titer was quantified by plaque assay. Several aliquots of 200 μL were made and kept at −80 °C.

Obtaining and culture of primary human monocytes and monocyte-derived macrophages (MDM).

Fifty mL of peripheral blood from 4 healthy individuals was taken in 2% EDTA tube and centrifuged at 500 RFC (R7

elative Centrifugal Force) / 10 min. The blood was diluted 1:1 with PBS (phosphate-buffered saline) 1X (Sigma Aldrich, MO, USA) and was deposited on a ten mL of lymphoprep density gradient (STEMCELL, CA, USA) as described previously [16], in line with the declaration of Helsinki. Thereafter was centrifuged at 800 RFC / 30 min; the interface corresponding to the peripheral blood mononuclear cells (PBMC) was recovered and subsequently three

washes were performed to eliminate platelets at 250 RFC / 10min, brake = 0. The percentage of CD14+ cells was then determined using a monoclonal mouse anti-human antibodies CD14-Fluorescein isothiocyanate (FITC, 2ng/ µl) (eBioscience, CA, USA) for 30 mn and was performed on a FACScanto flow cytometer (BD Biosciences). For this, 500,000 cells/well were seeded in 24-well plates with RPMI 1640 medium supplemented with 0.5% autologous serum [that was obtained from each individual and centrifuged at 2000 RPM (Revolutions per minute)/10 min], 1% antibiotics and left in culture for 2h at 37°C with 5% CO_2. Then, three washes were performed with PBS and RPMI 1640 medium supplemented with 10% of heat-inactivated Fetal Bovine Serum (FBS; Sigma Aldrich, MO, USA) and 1% antibiotics (Amphotericin B, Penicillin, Streptomycin; Sigma Aldrich, MO, USA).
Monocytes were used after overnight culture. To obtain monocyte-derived macrophages (MDM), the monocyte culture was led to differentiate for 6 days, changing culture media every 48h [16].

In vitro co-stimulation of monocytes and MDM with CHIKV and TLR agonists

The co-stimulation of monocytes and MDM was performed as follows: The CHIKV, MOI 10 and 5, for monocytes and MDM, respectively, was added simultaneously with Pam2CSK4 (5ng/ mL Monocytes; 20ng/mL for MDM), Poly (I:C) (20 µg/mL for both), LPS (10 ng/mL for monocytes; 20ng/mL for MDM) or R848 (5 µg/mL for both), agonists of TLR-2/6, -3, -4, and -7/8, respectively. However, previously we evaluated different concentrations of each agonist to determine the better concentration in monocytes since in macrophages the concentrations had previously been standardized in the group (unpublished data), and the TLRs activation in response to the agonist was determined based on the production of IL-6 and TNF-α. Based on those results obtained, we determined the concentration used in the present study. For primary cell cultures a volume of 200 µL was completed with RPMI 1640 medium, incubated for 1½ hours at 37°C and 5% CO_2. Thereafter, the medium was removed, and washes were performed with 1X PBS and replaced with 800 µL of RPMI 1640 medium supplemented with 10% SFB and 1% antibiotics. Supernatants and cells were collected at 6, 24, 48 hours post-infection (h.p.i.) for both primary cells culture.

Plaque-based assays

The plaque assay method was performed as follows: $1.5x10^5$ VERO /well cells were seeded in DMEM-1X medium (Sigma Aldrich, MO, USA) at 2% SFB, 1% Antibiotics. Serial 10 dilutions the supernatants of monocytes and MDM (10^1, 10^2) were made of by duplicate; the CHIKV infection was performed for 1½ hours, after which time the medium was removed and 800 µL of plating medium (DMEM-2X, 4% 2% carboxymethylcellulose SFB, 1% Antibiotics) was added and incubated at 37°C and 5% CO_2. After 2 days, the medium was removed, two washes were performed with PBS and violet crystal was added to reveal the lysis plates, and the viral titer was determined.

Quantification of IL-6 and TNF-α by ELISA

The collected culture supernatants of monocytes and MDM, both infected, were used to quantificated the IL-6 and TNF-α level production, through an ELISA test (BD OptEIA of BD Biosciences, CA, USA), following the manufacturer's recommendations. Briefly: The dishes were sensitized with the capture antibodies, overnight at 4°C; then washed with wash buffer (PBS 1X and 0.05% Tween-20) and after, blocking buffer (10% FBS and PBS 1X) was added of SFB) for 2 hours. Subsequently, the samples and standard recombinant cytokines (IL-6 and TNF-α) were added for 2h; after which, 3 washes were performed with PBS. Then, specific biotinylated detection antibody, together with avidin peroxidase, diluted in blocking buffer, was added and incubated for 1h at 25°C. Finally, TMB (3,3′,5,5′-Tetramethylbenzidine) was added for 15 minutes and the reaction was stopped with sulfuric acid (2 N). The colorimetric reaction was read on the Dynex Technologies, Inc. (Chantilly, VA, USA) spectrophotometer at a wavelength of 450 nm.

Total RNA Extraction

Total RNA extraction was extracted with TRIzol reagent (Invitrogen, Life Technologies, CA) and using the Direct-zol ™ RNA MiniPrep kit (Zymo Research, Irvine, California) following the manufacturer's instructions. The mRNA for IFN-β, PKR and OAS2 was quantified by real-time PCR, like this: A solution was made with Taq DNA polymerase (0.05 U/µL), MgCl2 (4mM), dNTPs (0.4mM), Nuclease-free water, reaction buffer (Mix Thermo, USA) and 0.6 µL of specific primers for each of the genes of interest were used (table 1);

Primers	
Interferón β (IFN β)	Forward. 5´-CGCCGCATTGACCATCTA-3´
	Reverse.5´GACATTAGCCAGGAGGTTTCTCA-3´
Protein kinase R (PKR)	Forward. 5´-GGTACAGGTTCTACTAAACA-3´
	Reverse. 5′-GAAAACTTGGCCAAATCCACC-3′
2'-5' Oligoadenylate synthase 2 (OAS2)	Forward. 5´-GTGTGTCCAAGGTGGTAAAGG-3´
	Reverse. 5´-CTGCTCAAACTTCACGGAA-3´
β-Actin	Forward.5´-ATCTGGCACCACACCTTCTACAATGA-3´
	Reverse. 5´-CGTCATACTCCTGCTTGCTGATCCAC-3´

Table 1. Primers used in the present study to quantify the interest genes

then, it was added in PCR tubes with 2 µL of the sample. Finally, the amplification and reading were performed in the Bio-Rad CFX .

Real-time PCR

The mRNA for IFN-β, PKR and OAS2 was quantified by real-time PCR, like this: A solution was made with Taq DNA polymerase (0.05 U/µL), MgCl2 (4mM), dNTPs (0.4mM), Nuclease-free water, reaction buffer (Mix Thermo, USA) and 0.6 µL of specific primers for each of the genes of interest were used (table 1); then, it was added in PCR tubes with 2 µL of the sample. Finally, the amplification and reading were performed in the Bio-Rad CFX .

Flow cytometry

FACSCanto II (Becton Dickinson, NJ, USA). We performed an exclusion of debris after which forward and side scattered light was used to identify cell populations (Monocytes) and measure the size and granularity of the cells. Cell auto-fluorescence was recorded by analyzing unstained cells in the FL1 channel (blue laser-488 nm and filter 530/30) as was previously described (17). To detect cell surface markers, treated cells were detached mechanically from the culture plates and incubated for 40 min at 4 ◦C in the presence of monoclonal mouse anti-human antibodies TLR-7-Fluorescein isothiocyanate (FITC; 2 ng/µL; clone: ABM2C27).

The samples were then washed and resuspended in PBS 1X and 10,000 events were recorded. Positive labeling cells were defined based on isotype-controls and a compensation matrix was performed to compensate for the spectral overlap. To quantify the percentage of CD14 positive cells from PBMCs, 5×10^5 PBMCs were incubated 20 min at 4 ◦C in the presence of monoclonal mouse anti-human CD14-FITC antibody (2 ng/ µL, clone: 61D3). Following incubation, the PBMCs were washed and resuspended in PBS 1X, and 10,000 events were recorded. For each experiment, unstained cells and conjugated isotype antibodies were included as controls. The expression of different markers was expressed as the mean fluorescent intensity (MFI) of the overall treated cells after subtraction of the isotype-control.

Statistics

Normality was tested by Shapiro-Wilk test. Friedman's test and Wilcoxon signed rank test post-hoc tests were used to observe the differences between the analyzed variables in GraphPad Prism 8.0.2. In all tests, values of $*p<0.05$ were considered significant.

Results

CHIKV Replication in monocytes

CHIKV infectious viral particle production was determined in infected monocyte supernatants, by plaque assay at 6, 24, and 48 h.p.i. The results (Fig. 1) show that the infectious viral particles begin to be detected after 6 h.p.i. ($3{,}3x10^3$ PFU/mL). The maximum peak of viral particle production was observed at 24 h.p.i. with an average production of $4{,}1x10^4$ PFU/mL and at 48 h.p.i. a rapid decrease in the production of viral particles ($1.3x10^3$ PFU/mL) was found, without compromising the cell viability. These results indicate that CHIKV replication is very rapid in monocytes and the fall in the production of viral particles drops drastically at 48 h.p.i. which suggests a possible antiviral response in infected monocytes.

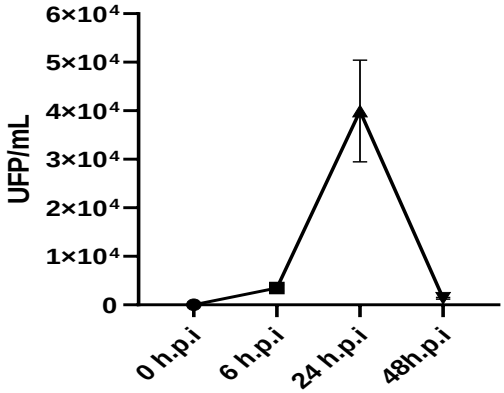

Figure 1. CHIKV replication kinetics in monocytes. The production of infectious viral particles was determined in supernatants of monocytes infected with CHIKV (MOI 10) at 6, 24, 48 h.p.i by plaque assay in Vero cells. Mean ± SEM.

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Triggering TLR-7/8 decreased CHIKV replication in monocyte

To determine the effect of TLRs activation with their respective agonists on CHIKV replication, a co-stimulus was performed and the release of infectious viral particles in the supernatants was determined by plaque-based assay at 6, 24 and 48 hpi. As we can observe in Figure 2, the activation of TLR-2/6, TLR-3, and TLR-4 induced a slight decrease in the production of infectious viral particles, compared to the control (monocytes without stimulation with agonists), but the difference was not statistically significant. The TLR-2/6, TLR-3 and TLR-4 activation effect on CHIKV infectious viral particle production was

observed from 6 h.p.i. Interestingly, treatment with R848, a TLR-7/8 agonist, statistically significantly decreased the production of infectious viral particles in supernatants of infected monocytes from 6 h.p.i. (Fig. 2). The greatest effect was observed at 48 h.p.i. since a lower production of viral particles was observed, in response to R848 treatment, compared the control (infected monocytes without treatment). That is, it went from $3{,}3x10^3$ to $1{,}2x10^3$ PFU/mL; of $4{,}1\ x10^4$ to $1{,}3x10^4$ PFU/mL and $1{,}3x10^3$ to $4{,}6x10^2$ PFU/mL at 6, 24 and 48 h.p.i respectively. Taken together, these results indicate that activation of TLR-7/8 rapidly decreased the CHIKV replication since the effect was observed from 6 h.p.i. and was maintained during 24 and 48 h.p.i. and at this last time, the effect was greater. At 48 h.p.i, there was a strong decrease in the production of infectious viral particles in infected monocytes without treatment (control); although, the decrease was greater in infected monocytes stimulated with R848.

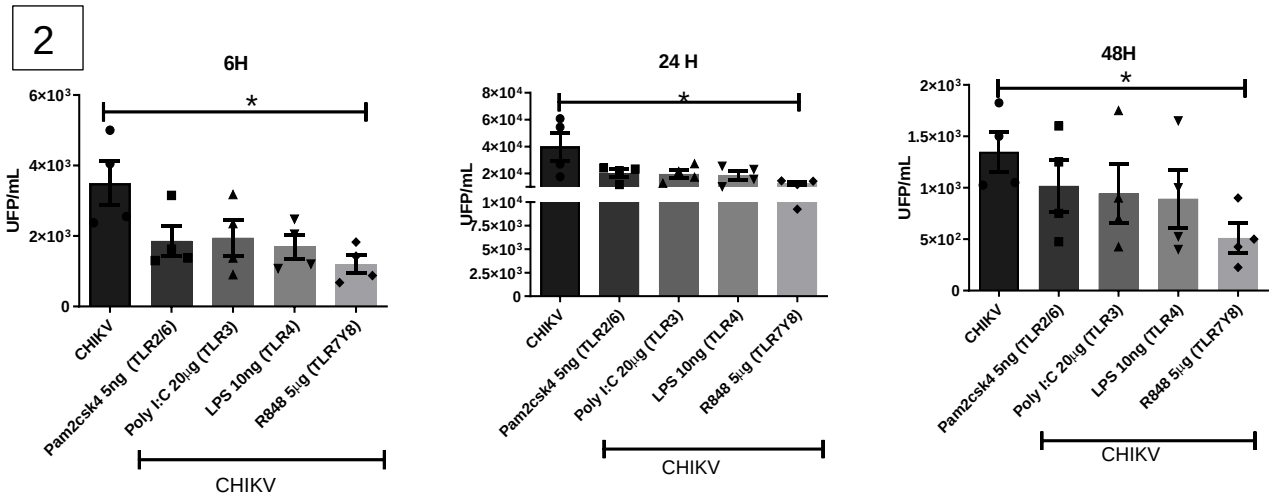

Figure 2. Activation of TLR-7/8 with R848 agonist decreases the production of CHIKV viral particles in monocytes. Monocytes were stimulated with Pam2CSK4 (5ng/mL), Poly (I:C) (20µg/mL), LPS (10ng/mL), and R848 (5µg/mL), agonists of TLR-2/6, TLR-3, TLR-4 and TLR-7/8, respectively and infected with CHIKV (MOI 10). At 6, 24, 48 h.p.i. the CHIKV infectious particles were determined by plaque assays in Vero cells.

Mean ± SEM. * p = <0.05.

Effect of TLR activation on TNF-α production in monocytes infected with CHIKV

TNF-α production was quantified by ELISA in monocyte culture supernatants with/without TLR agonists stimulation and infected with CHIKV. As we can observe in Fig. 3, the maximum production peak of TNF-α in monocytes infected with CHIKV without TLR activation occurs at 6 h.p.i. Although no significant changes in TNF-α production were observed in monocytes infected with CHIKV and stimulated with TLR-2/6, TLR-3 and TLR-4 agonists, if we found an increase, both at 6, 24 and 48 h.p.i. in the production of that cytokine, in monocytes infected and stimulated with R848, but a statistically significant increase was observed only at 48 h.p.i. (4362 pg/mL) compared to the monocyte infected with CHIKV without stimulation with TLR agonists (control) (930 pg/mL). (Fig. 3). However, the maximum production peak of this cytokine is at 6 h.p.i. (2550 pg/mL) in monocytes infected with CHIKV, time after which the production decreases or remains constant, until 48 h.p.i.

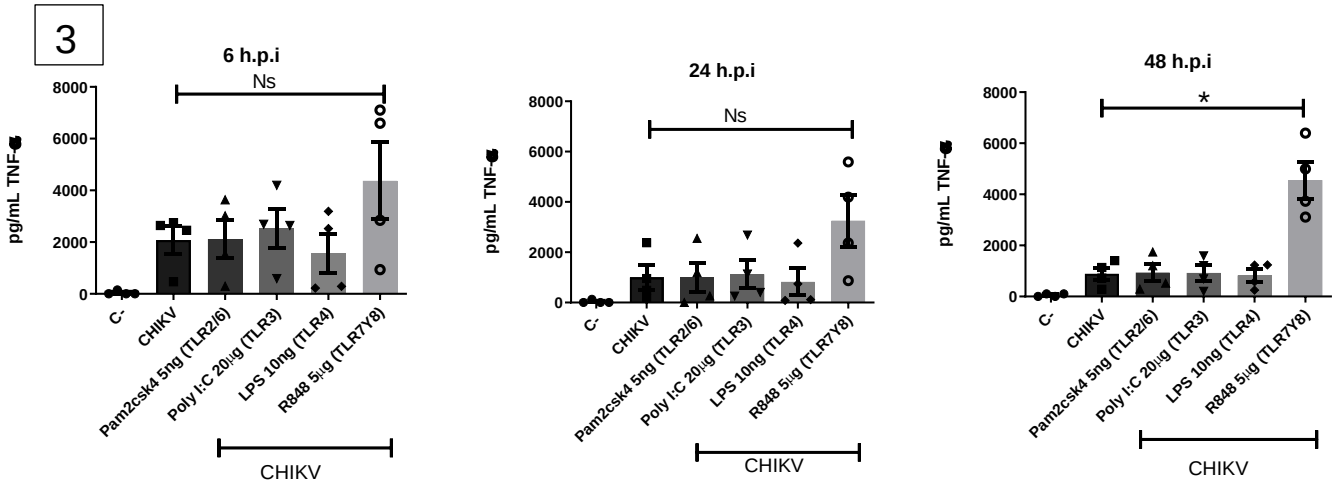

Figure 3. TNF-α production in supernatants of monocytes infected with CHIKV and co-stimulated with TLR agonists. Human monocytes were treated with agonists for TLR-2/6, TLR-3, TLR-4 and TLR-7/ 8 and infected with CHIKV (MOI 10). The TNF-α production was quantified in culture supernatants at 6, 24, 48 h.p.i. by ELISA. Mean ± SEM. * p = <0.05.

Effect of TLR activation on IL-6 production in monocytes infected with CHIKV

Besides TNF-α, we also quantified the production of IL-6 in the monocyte culture supernatants with/without TLR agonist stimulus and infected with CHIKV, by ELISA. As seen in Figure 4, the high production of IL-6 was observed at 6 and 24 h.p.i. but the maximum

peak of production was observed at 48 h.p.i. and that increase was statistically significant (4760 pg / mL) compared to monocytes infected only with CHIKV (2458 pg/mL) (control). In monocytes stimulated with agonists of TLR-2/6, TLR-3 and TLR-4, no changes in IL-6 production were observed compared to the control.

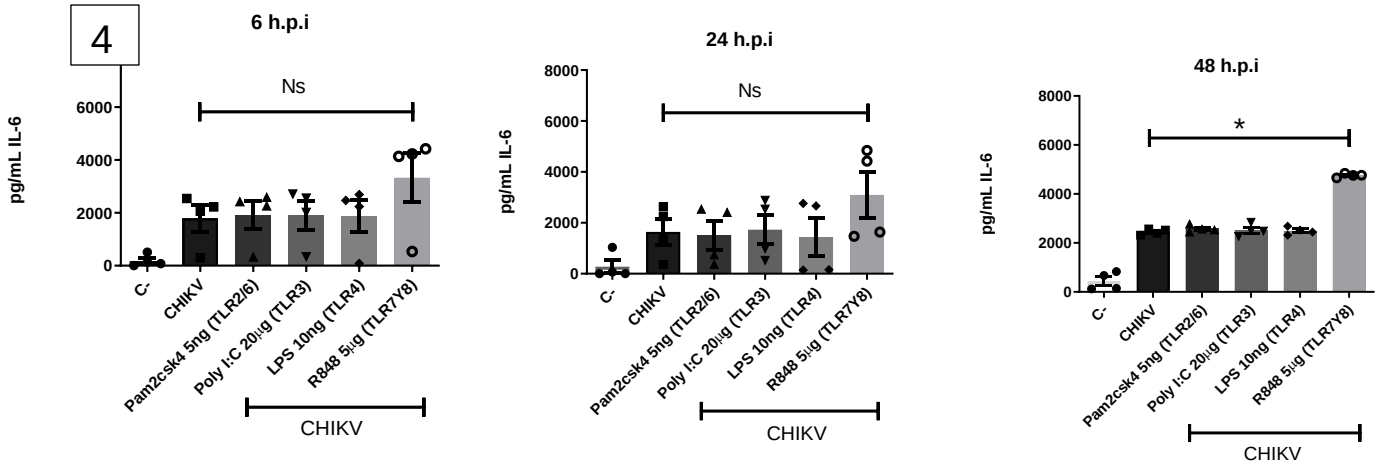

Figure 4. IL-6 production in supernatants of monocytes infected with CHIKV and co-stimulated with TLR agonists. Human monocytes were treated with agonists for TLR2/6, TLR3, TLR4 and TLR7/ 8 and infected with CHIKV (MOI 10) . IL-6 production was quantified in culture supernatants, at 6, 24, 48 h.p.i. by ELISA. Mean ± SEM. * p = <0.05.

Activation of TLR-7/8 induces the expression of IFN-I and IFN-stimulated genes

Since at 48 h.p.i. a strong decrease in the production of infectious CHIKV particles was observed in infected monocytes and treated with TLR-7/8 agonist, next step was to determine whether monocyte infection with CHIKV induced a type I IFN-dependent response. Therefore, the accumulation of mRNA for IFN-β and ISGs, such as PKR and OAS2, were quantified. Quantification was performed at 6, 24 and 48 h.p.i. by real-time PCR. Based on the results observed on TLRs activation, this evaluation was only carried out in monocytes infected with CHIKV and co-stimulated with R848, because only with this agonist we observed significant differences, both in the production of infectious viral particles and in the accumulation of pro-inflammatory cytokines, compared to the control. As shown in Figure 5A, stimulation with R848 induced the transcription of INF-β and the highest level of mRNA was observed at 6 h.p.i. (Fold change = 5,3), compared with monocytes infected with CHIKV

(Fold change = 1,5). At 24 h.p.i. a decrease of mRNA synthesis for INF-β was found and was similar to the control, but at 48 h.p.i., CHIKV infection induces a slight increase in the IFN-β mRNA. These results indicate that, in response to CHIKV infection monocytes implant a strong and rapid IFN production, whose expression is dependent on the activation of TLR-7/8 activation. Since this response may lead to the expression of ISGs, involved in antiviral response, our next step was to quantify the levels of OAS2 and PKR mRNAs.

It was found that the maximum PKR mRNA peak occurred at 24 h.p.i. in monocytes co-stimulated with R848 and infected with CHIKV (Fold change = 4,7) vs infected monocytes without further stimulation (Fold change = 1,0; Fig 5B). At 6 and 48 h.p.i. we did not find a difference in the PKR mRNA levels.

Concerning the OAS2 mRNA level, the maximum production peak was at 6 and 24 h.p.i. (Fold change =4,9) and it remained until 48, however it was not significant (Fold change = 3,8) compared to the control, monocytes only infected with CHIKV (Fold change = 1,0) (fig 5C).

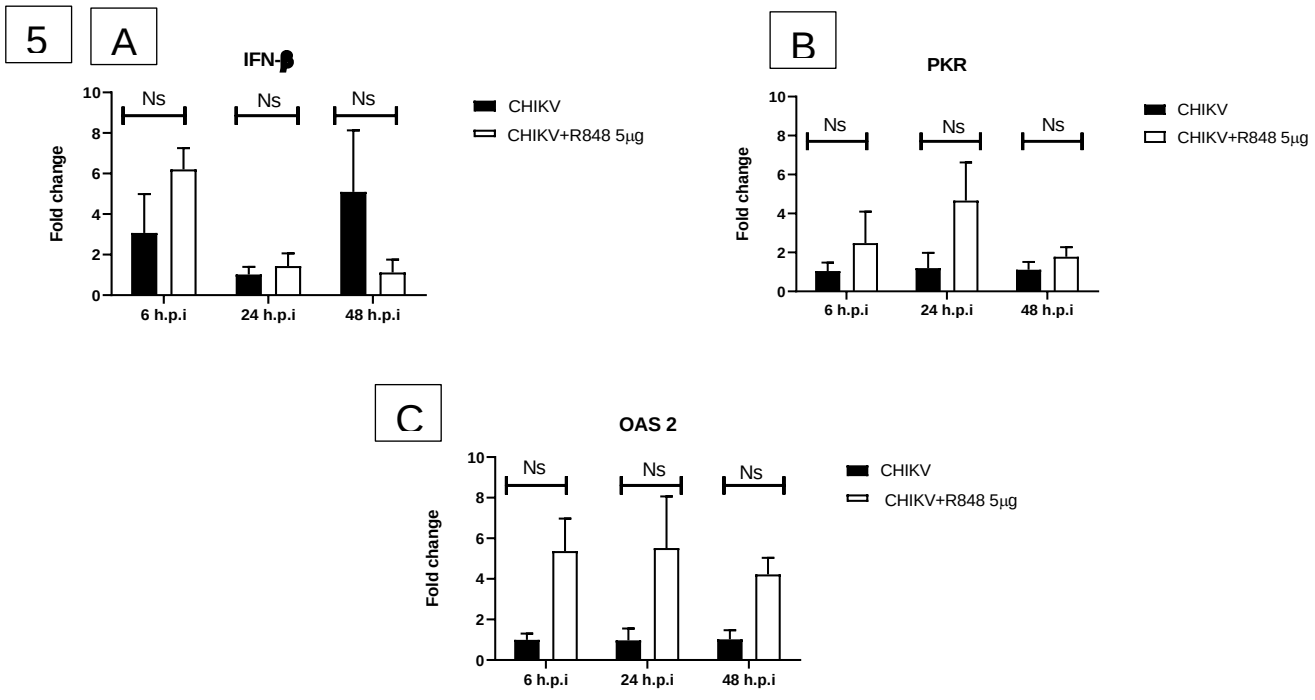

Figure 5. The activation of TLR-7/8 by its agonist induces the expression of IFN-β and ISG in monocytes infected with CHIKV (MOI 10) and co-stimulated with TLR agonists. At 6, 24, 48 h.p.i.

cells were harvested and total RNA extraction was done. Then, a DNA copy (cDNA) was synthesized and the mRNA levels for IFN, PKR, and OAS2 were quantified by real-time PCR. Mean ± SEM.

TLR 7 expression in monocytes stimulated with R848 and infected with Chikungunya virus.

The expression of TLR-7 was determined for cytometry flow at 6, 24, and 48 h.p.i. The results (Fig. 6) show at 6 h.p.i., a mean fluorescent intensity (MFI) similar between monocytes no infected (403,5 MFI), monocytes infected (235,5 MFI) and monocytes co-stimulated with R848 (357); however, in the last group is slightly slow the expression. An increase in the expression was observed at 24 h.p.i in the groups, for monocytes no infected (650 MFI), monocytes infected (728 MFI) and monocytes co-stimulated with R848 (503 MFI), suggesting that CHIKV infection induces an increase in the TLR-7 expression but no-statistically significant, which corresponds to the peak of infectious viral particles. At 48 h.p.i the MFI was 620 for no infected monocytes; in infected monocytes the MFI was 359 and in monocytes infected and treated with R848, the MFI was 481.

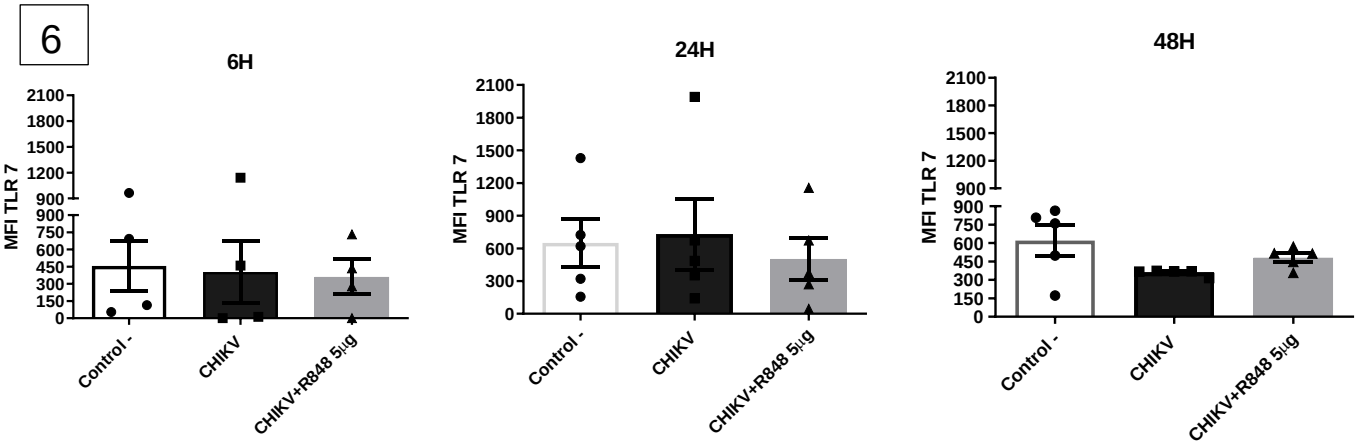

Figure 6. TLR 7 expression in monocytes infected with CHIKV and co-stimulated with R848 agonist. MDM were stimulated with R848 (5µg/mL) an agonist of TLR-7/8 and infected with CHIKV (MOI 5). At 6, 24, 48 h.p.i. cells were harvested, labeled with FITC- anti TLR-7 and analyzed by flow cytometry. Mean ± SEM.

CHIKV Replication in monocytes-derived macrophages (MDMs)

CHIKV infectious viral particle production was determined in infected MDMs supernatants, by plaque assay at 6, 24, and 48 h.p.i. The results (Fig. 7) show that the infectious viral particles begin to be detected after 6 h.p.i. ($4{,}1x10^3$ PFU/mL). The maximum peak of viral

particle production was observed at 24 h.p.i. with an average production of $2{,}0x10^4$ PFU/mL at 48 h.p.i. a rapid decrease in the production of viral particles ($5x10^3$ PFU/mL) was found, without compromising the MDMs viability. These results indicate that CHIKV replication is very rapid also in MDM, like monocytes, and the fall in the production of viral particles drops drastically at 48 h.p.i. which also suggest a possible antiviral response. However, our results suggest that MDMs are less producers of viral particles.

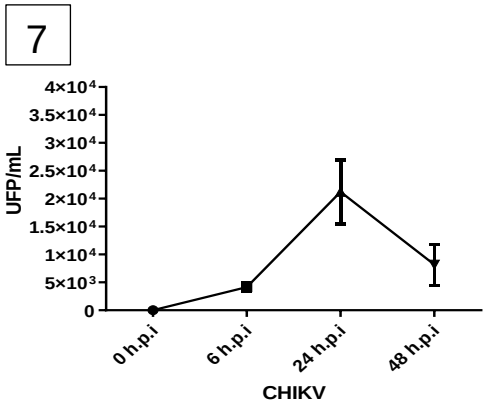

Figure 7. CHIKV replication kinetics in MDM. The production of infectious viral particles was determined in MDMs culture supernatants infected with CHIKV (MOI 5) at 6, 24, 48 h.p.i by plaque assay in Vero cells. Mean ± SEM.

Effect of triggering TLRs MDM in the CHIKV replication

To determine the role of TLRs on CHIKV replication in MDMs, the TLRs were activated with their respective agonists and simultaneously infected with CHIKV. Then, the release of infectious viral particles in the cultures supernatants was performed by plaque-based assay at 6, 24, and 48 hpi. As we can observe in Figure 8, the activation of TLR-4, and TLR-7/8 induced a slight decrease in the production of infectious viral particles, compared to the control (MDMs without stimulation with agonists) at 6 and 24 h.p.i. but without statistically

significant. That is, with TLR-4 it went from 4,1 x10^3 to 2,9 x10^3 PFU/mL; 1x10^4 to 1,8x10^4 PFU/mL; 8,1x10^3 to 6,5 x10^3 PFU/mL at 6, 24 and 48 h.p.i. respectively, comparing MDM infected without and with TLRs activation. For TLR- 7/8 it went from 4,2x103 to 2,7 x103 PFU/mL; 1x10^4 to 1,7x10^4 PFU/mL ; 8,1x10^3 to 6,8 x10^3 PFU/mL However, at 48 h.p.i. we did not observe a decrease in the replication of CHIKV compared to the infection control, suggesting that TLRs activation have not effect on CHIKV replication in MDMs.

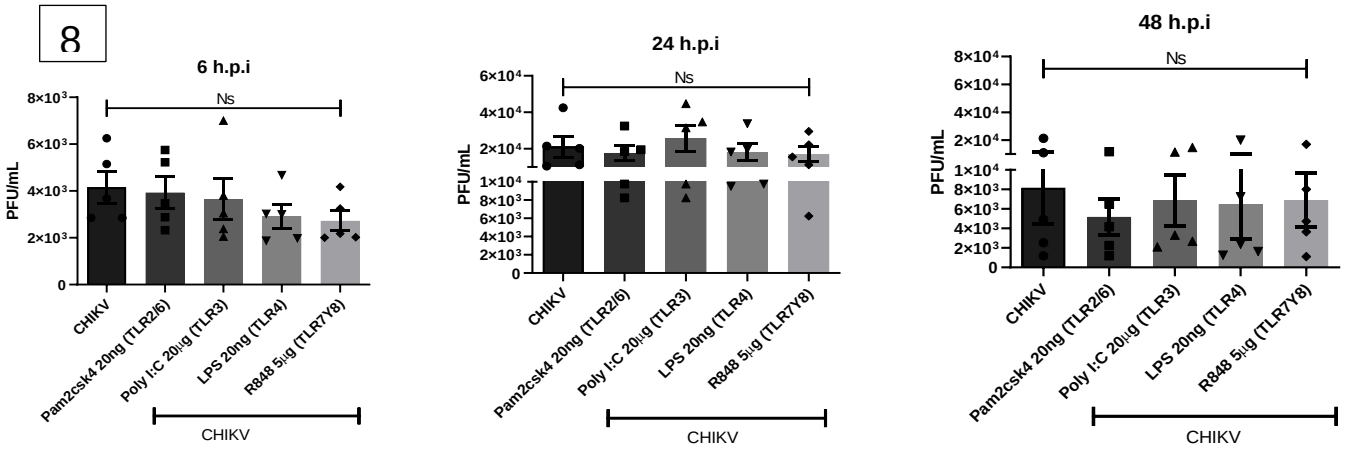

Figure 8. Effect of triggering TLRs in the production of CHIKV viral particles in MDM. MDM were stimulated with Pam2CSK4 (20ng/mL), Poly (I:C) (20µg/mL), LPS (20ng/mL), and R848 (5µg/mL), agonists of TLR-2/6, TLR-3, TLR-4 and TLR-7/8, respectively and simultaneously infected with CHIKV (MOI 5) a t 6, 24, 48 h.p.i. The CHIKV infectious particles were determined by plaque assays in Vero cells. Mean ± SEM.

Effect of TLR activation on TNF-α production in MDMs infected with CHIKV

TNF-α production was quantified by ELISA in MDMs culture supernatants with/without TLR agonists stimulation and infected with CHIKV. As we can observe in Fig. 9, the maximum production peak of TNF-α in MDMs infected with CHIKV without TLR activation occurs at 6 h.p.i. Although no significant changes in TNF-α production were observed in MDMs in response to CHIKV infection and stimulated with TLR-2/6, TLR-3, we did find a decreased non- significant in the production of TNF-α at 6 and 24 48 h.p.i. in MDMs infected and stimulated with TLR4, compared to the MDMs infected with CHIKV without stimulation with TLR-4 agonists (control). A strong decreased in TNF-α production was

observed at 48 h.p.i. in MDM infected with or without TLR activation and the accumulation was similar in the two cases.

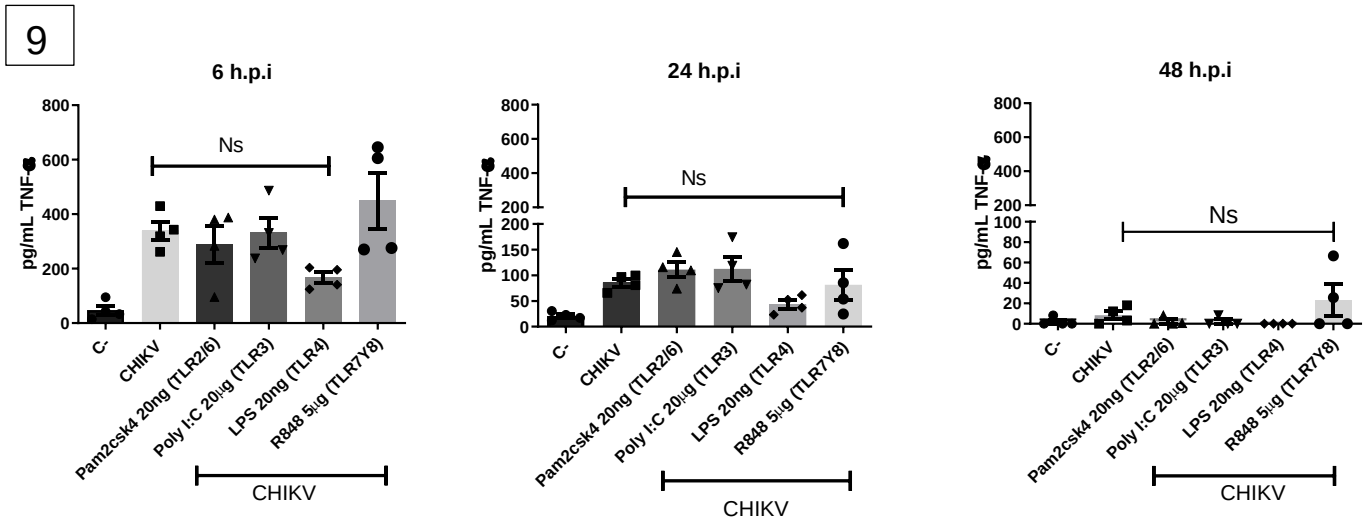

Figure 9. TNF-α production in the MDMs culture supernatants infected with CHIKV and co-stimulated with TLRs agonists. Human MDMs were treated with agonists for TLR-2/6, TLR-3, TLR-4 and TLR-7/ 8 and infected with CHIKV (MOI 5). The TNF-α production was quantified in culture supernatants at 6, 24, 48 h.p.i. by ELISA. Mean ± SEM.

Effect of TLR activation on IL-6 production in MDMs infected with CHIKV

Besides TNF-α, we also quantified the production of IL-6 in the MDM culture supernatants with/without TLRs agonist stimulus and infected with CHIKV, by ELISA. As seen in Figure 10, the high production of IL-6 was observed at 24 and 48 h.p.i. and the level accumulation was similar in MDMs with and without TLRs activation and infected. However, the highest level was observed in response to TLR-7/8 activation in the 3 points evaluated, although it not statistically significant.

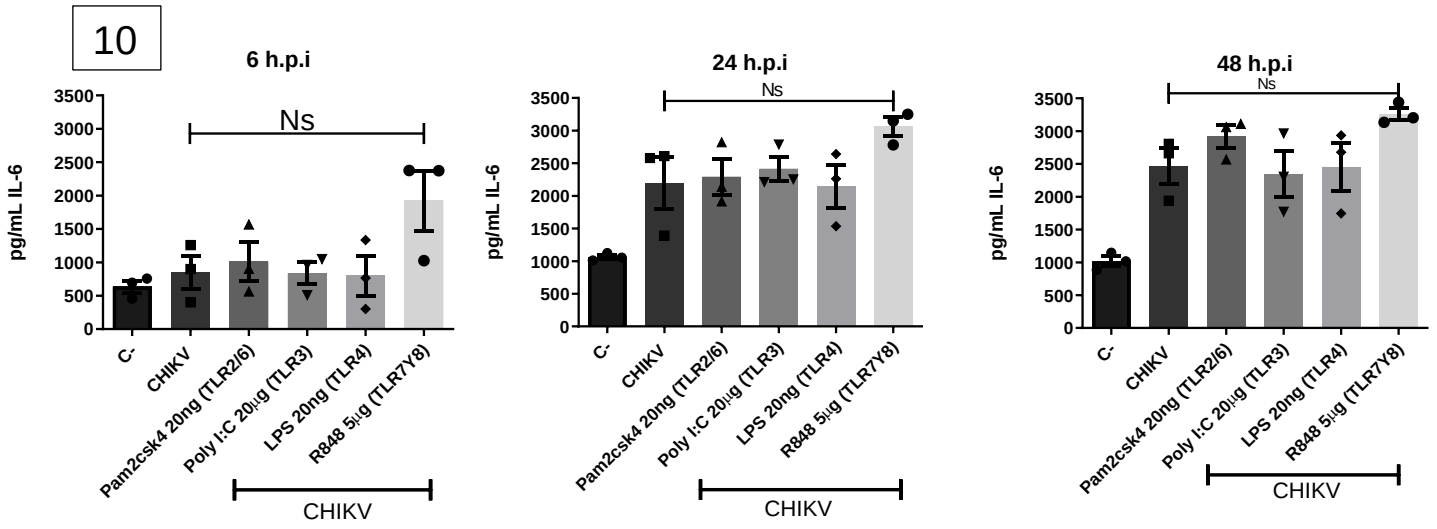

Figure 10. IL-6 production in supernatants of MDMs cultures infected with CHIKV and co-stimulated with TLR agonists. Human MDM were treated with agonists for TLR-2/6, TLR-3, TLR-4 and TLR-7/ 8 and infected with CHIKV (MOI 5). IL-6 production was quantified in culture supernatants at 6, 24, 48 h.p.i. by ELISA. Mean ± SEM.

Discussion

The more common clinical manifestation of CHIKV infection is the arthropathy that generally is viewed as an immunopathology, likely initiated by direct infection of tissues like joints, liver or SNC. A severe immune defense reaction with increased proinflammatory cytokines is responsible for joint inflammation, with recruitment of the pro-inflammatory cell to the site of infection, such as monocytes and macrophages. It has been reported in mice and humans infected that these cells are associated with immunopathogenesis [18][11]. Nevertheless, it has not yet been clear if the primary human monocytes or macrophages are targets of infection by CHIKV [19][20]. In the present study we determine a productive infection in the monocytes and MDM, with release of infectious viral particles from 6 h.p.i with a maximum peak at 24 h.p.i. However, at 48 h.p.i. The viral titers decreased drastically. Based on these results we hypothesized that monocytes and MDM might be involved in the viral clearance.

The recognition of viral PAMPs in the site of infection, by PRRs, that lead to the establishment of an inflammatory response is an essential process of the host innate immune response against pathogens [21]. Monocytes, macrophages and dendritic cells, are part of the

host's innate immune response and this could explain why they are one of the first cells to be recruited to the site of infection and are involved in the viral recognition. This they do through the PRRs, includingTLR-3, TLR-7 and TLR-7/8 that can be activated thanks to viral replication intermediaries, such as dsRNA (TLR-3) or ssRNA that is recognized by TLR-7/8 or RIG-I/MDA-5.

Have been reported that TLR-3 is a critical PRR in the control of CHIKV replication, in the immunity response and pathology both humans and mice, since in knockout mice for TLR-3, greater viral titers and greater myeloid cell infiltration were observed, which corresponds to more severe arthritic symptoms compared to control mice [21]. In other study conducted in mouse neuronal cells, high expression of TLR-3 was reported, whose activation leads to the elimination of CHIKV from 9 to 10 d.p.i. [22]. Or, when mice were pretreated with Poly I:C, a reduction of CHIKV titer in the brain and the 100% of the animals were protected. In a study conducted in bronchial cells treated with Poly I:C, a lower cytopathic effect was observed in the cells due to inhibition of CHIKV replication [23]. However, in the present we did no observed a decreased in the viral titer in primary human monocytes and MDMs both infected with CHIKV and treated with Poly I:C, compared with cells infected with CHIKV and without poly I:C treatment. This may be due to monocytes express low levels of TLR3. as we observed by qPCR (data not shown). Contrary to our results, previously was reported in MDMs an induction of TLR- 3 in response to CHIKV infection [24]; this may be due to time of stimulation with the TLR-3 agonist.

Similarly, in a study conducted in patients, it was found that polymorphisms including rs179010, rs5741880, rs3853839 for TLR-7 and the polymorphism rs3764879 for TLR-8 increases susceptibility to CHIKV infection with more severe clinical symptoms, when compared to control patients [25]. In a study conducted in mouse neuronal cells, positive regulation of TLR-7 was evidenced after infection with the CHIKV, which may contribute to the clearance of the infection in mice with neuronal involvement [26]. In other study using Ross River virus (RRV) as model, another alphavirus arthritogenic, was observed that TLR-7 may contribute to the early antiviral response [27]. In human monocytes infected with the Zika virus (ZIKV) and stimulated with R848, was found that TLR-/8 activation blocks ZIKV replication in myeloid cells at micromolar concentrations [28]. Similarly, we found that the monocytes infected with CHIKV and co-stimulated simultaneously with R848, releases

lower infectious viral particles starting at 6 h.p.i whose effect is maintained a. 24 and 48 h.p.i., that monocytes without TLR-7/8 activation. However, this antiviral activity TLR-7/8 dependent was not observed with the same intensity in CHIKV-infected MDM. These results suggest a possible antiviral response dependent of TLR-7/8 activation and cell population.

Although TLR-2/6 and TLR-4 are not classically activated by CHIKV replication intermediaries or viral genome, recently it was reported that these two TLRs were activated in response to viral infection. e.g. an activation of TLR-2/6 by DENV NS1 protein; in addition, an up-regulation in the expression of those TLRs was observed in DENV-infected human PBMC [29]. Furthermore, TLR-4 can be activated by the respiratory syncytial virus fusion protein [30][31]. However, it has not yet been documented that these two TLRs are activated in response to infection with CHIKV. Therefore, in the present study, we intended to observe if both TLRs were involved in the recognition and control of viral replication. Although a quick reduction in the infectious viral particles releases was observed, mainly at 6 h.p.i. it was not statistically significant compared with the releases in monocytes and MDM infected but without activation of TLR-2/6 and TLR-4. Therefore, future studies that can elucidate the role of both TLRs in the recognition and control of CHIKV infection in monocytes and MDMs should be carried out.

Once activated the TLRs signal through adapter molecules such as MyD88, the activation signal of TLR-dependent pathway leads in the translocation of the transcription factor Nf-κB to the nucleus and induces the production of proinflammatory cytokines, such as IL-6, IL-8, TNF-α, IL-1β, IRF3 and IRF7. Furthermore, the activation of IRF3 and IRF7 signaling cascade result in the production of type I IFNs, i.e. IFN-α/β [32],[33],[13]. In murine models, the role of IFN-I in the control of viral replication has been documented. In fact, both in vitro models, with cell lines, and in in vivo with murine models, the antiviral activity of IFN-I against CHIKV has been observed, which translates into the reduction of viral titers and the reduction of symptoms, such as edema and arthralgia [34][35]. Although IFN-α/β signals through the same receptor, in a study using knockout mice for IFN-α or IFN-β, it was found that although both have antiviral activity against CHIKV, these act through different mechanisms. IFN-α limits CHIKV replication and dissemination, whereas IFN-β protects from CHIKV pathogenesis by limiting inflammation mediated by neutrophils [36]. The IFN-I can trigger the activation of a specific signal transduction pathway leading to induction of

ISGs expression that are responsible for the establishment of an anti-viral state. Binding enzymatically OAS active to the viral RNA results in the production of 2′- 5′ oligoadenylate synthetases (2-5OAS). Latent monomeric RNase L is enzymatically activated through homodimerization induced by binding to 2-5OAS oligomers. Once activated, RNase L degrades ssRNA molecules, including mRNA and viral RNA. In a study conducted in HeLa cells expressing OAS3, the decrease in viral particles of CHIKV was observed [37][38]. Furthermore, a study conducted in patients infected with CHIKV and healthy controls, it was observed that polymorphisms in the OAS gene can increase susceptibility to CHIKV infection and may increase the risk of chronic symptoms [39]. PKR is also part of the ISGs, and like OAS, PKR also has the antiviral activity. In this case, the PKR mechanism is related to the ability of this enzyme to control mRNA translation, through phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF2α)[40][41]. However, in a study performed on fibroblasts reported that CHIKV infection leads to PKR-dependent phosphorylation of eIF2α, but that this process is not essential to translational shutoff [42]. Similarly, this study shows a high expression of IFN-I mRNA, in monocytes stimulated with R848 and infected compared to CHIKV infection only. According with these results, here we also observed that CHIKV infected monocytes and stimulated simultaneously with R848, not only does it lead to a decrease in viral titles, but also to an increase OAS2 mRNA lev-0els at 48 h.p.i. The increase in the expression of the PKR and OAS mRNA could be a consequence of the activation of TLR-7/8, resulting in a slight increase in the levels of IFN-I, mainly at 6 h.p.i. which at the same time, induces the expression of these two ISGs evaluated. So, it can be suggested that OAS-2 exerts the greatest effect on decreasing the replication viral, since the OAS-2 mRNA expression was started at 6 and stayed until 48 h.p.i. Since the increase in mRNA levels of PKR and OAS-2 coincides with the decrease in CHIKV titers, which begins at 24 h.p.i., we suggest that both PKR and OAS2 could be involved in the control of virus replication.

Inflammatory cytokines and chemokines are thought to be involved in the pathogenesis of CHIKV and are associated with severe clinical presentations as well as with the development of abrupt and persistent arthralgia [43]. previously was reported a significant increase of IL-1β and IL-6 levels in severe cases of chikungunya compared with non-severe cases [35]. An increase in these cytokines has also been seen in patients with chronic arthralgia compared

to control patients [44]. In this study, it was found that monocytes and MDM infected with CHIKV produce high levels of IL-6 and TNF-α compared to uninfected monocytes. Likewise, a greater production of these two cytokines was found in CHIKV infected monocytes simultaneously stimulated with R848, compared to monocytes, but in MDM the TNF-α was tendency decreased in response to TLR-4 activation. Our results are very interesting since a significant increase in TNF-α in patients with persistent arthralgia in comparison to patients who had fully recovered [45]. These results suggest that its level is positively correlated with CHIKV pathogenesis and severity of chikungunya fever. Furthermore, our results suggest that one of the major sources of TNF-α in response to CHIKV infection could be monocytes. Interestingly, the knocked down the expression of TNF-α using its specific siRNA prior to HCV infection increased the levels of intracellular HCV RNA as well as the level of the HCV core protein, compared with cells treated with the control siRNA [46], indicating that TNF-α could indeed suppress HCV replication. Therefore, an increase in TNF-α as we observed here, could be playing a role in the control of CHIKV replication, as was reported for HCV, likely due to its role in IFN signaling and the induction of ISGs [46].

This is important because these cytokines play an important role in the recruitment of pro-inflammatory cells, essential for the resolution/pathogenesis of arthritis. However, a hold inflammation has been also associated with the presentation of persistent arthralgia and chronicity of the CHIKV infection, so the possibility of using R848 as an adjuvant in vaccines should be studied further [45],[47].

Conclusion

Our study shows that monocytes stimulated with TLR-7/8 agonist R848 negatively regulate the production of viral particles from the earliest time (6 h.p.i.) to the latest time evaluated (48 h.p.i.), an increase in pro-inflammatory cytokines, (IL- 6 and TNF-α), and finally, we found a slight increases expression of IFN-β, as well as ISGs, including OAS2 and PKR.

In macrophages we did not find a decrease in replication with any of the stimuli with the TLRs agonists; however, we found a tendency decrease in TNF-α with the TLR 4 agonist and a tendency to increase both cytokines with the R848 stimulus.

This brings us closer to understanding the differential activation of TLRs in monocytes and MDMs, knowing that these cells participate in the pathogenesis and / or resolution of the infection.

It also sheds light on possible therapeutic targets that may lead to blocking CHIKV infection and / or controlling the exacerbated inflammatory response.

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