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Human keratinocytes restrict chikungunya virus replication at a post-fusion step

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ABSTRACT

Transmission of chikungunya virus (CHIKV) to humans is initiated by puncture of the skin by a blood-feeding *Aedes* mosquito. Despite the growing knowledge accumulated on CHIKV, the interplay between skin cells and CHIKV following inoculation still remains unclear. In this study we questioned the behavior of human keratinocytes, the predominant cell population in the skin, following viral challenge. We report that CHIKV rapidly elicits an innate immune response in these cells leading to the enhanced transcription of type I/II and type III interferon genes. Concomitantly, we show that despite viral particles internalization into Rab5-positive endosomes and efficient fusion of virus and cell membranes, keratinocytes poorly replicate CHIKV as attested by absence of nonstructural proteins and genomic RNA synthesis. Accordingly, human keratinocytes behave as an antiviral defense against CHIKV infection rather than as a primary targets for initial replication. This picture significantly differs from that reported for Dengue and West Nile mosquito-borne viruses.

Introduction

The skin is at the interface of vector-to-human transmission of mosquito-borne viruses. The transmission cycle of these pathogens is initiated when virus-containing fluids are ingested by a blood feeding vector by skin puncture of an infected vertebrate. Once the virus has replicated in the insect vector, it reaches the salivary glands where replication leads to the presence of high infectious titers (Luplertlop et al., 2011; Salazar et al., 2007; Vazeille et al., 2010; Ziegler et al., 2011). During a subsequent blood meal, skin probing by the proboscis of the infected mosquito results in the extravascular delivery of the virus in both the epidermis and dermis.

The human skin is a complex organ composed of multiple cell types. Besides its role of physical barrier against environmental aggressions, the presence of resident and migratory immunocompetent cells gives the human skin a key role in the detection and defense against pathogens. The capacity of mosquito-borne viruses to replicate in cells at the anatomical site of mosquito bite which implies counteracting innate immune responses in these cells, together with the encounter of competent cells with migratory properties will determine the establishment of a systemic infection and the continuation of transmission cycles between the vertebrate host and the arthropod vector (for revue see (Briant et al., 2014)). Keratinocytes that represent the major cell population in the epidermis perfectly illustrate the dual role of the skin upon virus inoculation. Indeed, these cells present in the outermost cornified skin layer as well as in the deeper epidermis not only contribute to the substructure of the skin but also fulfill a key role in the detection and control of pathogens facilitated by the expression of pattern recognition receptors (Nestle et al., 2009). In the recent years, we, and others, demonstrated that keratinocytes are primary targets for initial replication of arboviruses sharing the common property to be inoculated in the vertebrate skin by blood-feeding mosquitoes. Indeed, keratinocytes efficiently replicate Dengue virus (DENV) and West Nile virus (WNV) and this cell type was therefore proposed as a major player for host colonization by *flaviviruses* (Lim et al., 2011; Limon-Flores et al., 2005; Surasombatpattana et al., 2011). Both viruses also stimulate the

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transcriptional activation of intracellular RNA virus sensors, IFN genes and antimicrobial proteins attesting for the establishment of antiviral innate immunity in this cell type (Surasombatpattana et al., 2011).

Similarly to DENV and WNV, the chikungunya *alphavirus* (CHIKV) (family *Togaviridae*) is transmitted to humans through skin puncture by infected mosquitoes. In humans, CHIKV replication is responsible for an acute syndrome lasting for 3–7 days characterized by a febrile illness, headache, joint pain, myalgia and by a cutaneous rash in 30–40% of infected patients (Schwartz and Albert, 2010). Hallmarks of acute CHIKV infection also rely on the elicitation of a strong immune response involving the production of proinflammatory mediators including TNF-α/β, IFN-α,-β and -γ, IL-4 and IL-10 and MCP-1 in tissues (Rulli et al., 2007; Thangamani et al., 2010; Wauquier et al., 2011). In CHIKV-challenged mice a burst of IFNs production occurs locally at the site of inoculation (Schilte et al., 2010). Histological analysis of tissues collected from these animal or from experimentally inoculated macaques revealed that the presence of CHIKV antigens and RNA in the skin is preferential in fibroblasts (Couderc et al., 2008; Labadie et al., 2010; Rudd et al., 2012; Schilte et al., 2010) while its presence in keratinocytes has never been reported so far. The present study was therefore, designed to determine the behavior of human keratinocytes upon CHIKV challenge. Here, we show that infection of keratinocytes elicits a strong antiviral program attested by the significant transcriptional activation of pattern-recognition receptors genes together with increased type I, II and III interferons mRNA levels. Consistent with these data we report that human primary and immortalized keratinocytes can be infected by CHIKV as attested by viral particles endocytosis and fusion of the viral envelope glycoproteins with cellular membranes. However, viral RNA synthesis was impaired in these cells and *de novo* viral particle release could not be detected attesting for an intracellular block of CHIKV replication in human keratinocytes. Altogether, our data evidence that keratinocytes behave as key defense against CHIKV in the skin and provide new insight into the picture of early events of CHIKV infection, a pathogen that continues spreading across the Caribbean islands (Van Bortel et al., 2014) and represents a major public health risk in many parts of the world, including in temperate areas colonized with the appropriate strains of competent mosquitoes (Angelini et al., 2008; Vazeille et al., 2008).

Results

Primary and immortalized human keratinocytes are poorly permissive for CHIKV replication

First, we investigated the capacity of human keratinocytes to support CHIKV replication. To this end, primary human epidermal keratinocytes obtained from human foreskins (referred below as NHEK) or the HaCaT cell line, a spontaneously immortalized human keratinocytes cell line representing a relevant model to study keratinocytes in vitro (Boukamp et al., 1988), were challenged with CHIKV-LR-3'GFP viruses encoding a GFP reporter gene at the 5′ end of structural proteins (Tsetsarkin et al., 2006). Cultures of HEK293T human kidney epithelial cells, referred below as 293T, well known as CHIKV-permissive cells (Bernard et al., 2010) were processed in similar conditions and used as a positive control. Viral replication was monitored over time by detection of GFP expression in the culture. As GFP is not incorporated into virions produced with the CHIKV-LR-3'GFP subgenomic clone, green fluorescence in the culture provides direct evidence of viral replication (Vanlandingham et al., 2005). After 24 h in culture, approximately 60% of 293T cells exposed to a MOI of 1 were GFP-positive (Fig. 1A). GFP expression reached 80% after 48 h. In contrast, below 2% GFP-positive cells were detected in cultures of NHEK and HaCaT cells at any time. These rare GFP-positive cells rapidly underwent apoptosis as attested by caspase-8/-9 detection (Supplementary Figure 1). No sign of viral spreading in the culture was detected even when these cells were exposed to higher multiplicity of infection (MOI=50) or when cultured for eight days (data not shown). Similar results were finally obtained using immortalized SCC12B2 and SCC12F2 squamous carcinoma keratinocytes with distinct differentiation stages as well as using adult primary human keratinocytes (data not shown). The absence of CHIKV replication in primary and immortalized keratinocytes was not the result of a general refractory state since keratinocytes maintained in our culture conditions were successfully infected with Dengue virus or West Nile virus (Supplementary Figure 2) in agreement with previous observations (Lim et al., 2011; Surasombatpattana et al., 2011).

Alphaviruses, and especially CHIKV, produce a marked cytopathic effect in vertebrate cell cultures (Sourisseau et al., 2007). Here, we found that CHIKV used at a MOI of 1, decreased the viability of 293T by more than 20% and 60% cells at 48 and 72 h post-infection respectively (Fig. 1B). In identical culture conditions, poor cytopathic effect was observed in HaCaT and NHEK cells along the experiment, with more than 90% of the cells remaining unaffected by CHIKV after 72 h of cultivation. Next, we investigated progeny virions production in these cultures. NHEK or HaCaT cells were challenged with CHIKV for 2 h at 37 °C. After extensive washings and trypsin treatment to remove viral particles docked to the membranes, the cells were maintained in culture for an additional 24 h or 48 h. Then, *de novo* production of viral particles was monitored by qRT-PCR amplification of CHIKV RNA in culture supernatants. As shown in Fig. 1C, viral genomic RNA was detected at background levels in these samples while RNA copy numbers increased overtime to reach values above 10^7 copies/ml in supernatants of 293T cells processed in identical conditions. When incubated with fresh cultures of permissive 293T cells, supernatants of CHIKV-challenged NHEK or HaCaT from Fig. 1C were unable to produce GFP (data not shown), attesting for the absence of release of infectious particles. Altogether, these data show that human keratinocytes are unable to support productive infection after CHIKV challenge.

CHIKV particles are endocyted by HaCaT cells

In permissive cells, CHIKV infection is initiated by E2 envelope glycoproteins binding to an uncharacterized receptor and endocytosis of the viral particle into Rab5-positive endosomes where fusion occurs (Bernard et al., 2010). The capacity of CHIKV particles to be endocyted in keratinocytes was therefore examined by immunofluorescence detection of viral structural proteins. HaCaT cells were incubated with CHIKV (MOI=100) for 1 h at 37 °C. After extensive washings, the cells were permeabilized and stained with anti-capsid mAbs and with Alexa 647-labeled wheat germ agglutinin (WGA), a carbohydrate-binding lectin that recognizes sialic acid and N-acetylglucosaminyl sugar residues predominantly found in cellular membranes. Confocal fluorescence microscopy analysis (Fig. 2A) revealed the presence of capsid proteins at the cell periphery. Optical sections performed from a series of stack sections across *Z* axis and spectral analysis confirmed the presence of green fluorescent spots corresponding to capsid antigens in the cytoplasm, beneath the plasma membrane. No signal was evident in non-infected cells, confirming the specificity of the assay (Fig. 2A, left panel). The presence of intracytoplasmic capsids was further confirmed by electron microscopy analysis. HaCaT cells were challenged for 10 min with CHIKV used at a high infectious dose (MOI=500) in order to increase the probability to detect CHIKV particles in thin sections. Micrographs presented in Fig. 2B confirmed the presence of *bona fide* viral particles in the cytoplasm of immortalized keratinocytes. Next, the capacity of CHIKV capsids to colocalize with Rab5-positive endosomes was examined in HaCaT cells. Confocal

analysis of cells co-labeled with antibodies specific of capsid protein (green) and of Rab5 antigens (red) revealed the presence of a yellow/orange granular labeling in the cytoplasm of cells challenged with CHIKV for 30 min (Fig. 2C). The colocalization of green and red fluorescences in some areas was confirmed by spectral analysis. Quantitative analysis of colocalization events revealed that about 15%, of cell-associated CHIKV antigens detected in human keratinocytes colocalized with the early endosomal compartment. Similarly, 10 to 15% of intracytoplasmic capsids detected in CHIKV-infected 293T cells merged with Rab5 signal when processed in these experimental conditions (data not shown). According to these results, CHIKV particles were apparently endocyted and routed to early endosomes with similar efficiency in permissive 293T cells and HaCaT cells.

Keratinocytes membranes support fusion with CHIKV envelope glycoproteins

Next, we questioned the capacity of CHIKV envelope to fuse with cellular membranes in human keratinocytes using retroviral particles pseudotyped with CHIKV envelope glycoproteins. 293STAR cells (Ikeda et al., 2003) containing a stable retroviral vector expressing human immunodeficiency type 1 (HIV-1) Gag and Pol proteins allowing extracellular release of retroviral pseudoparticles (VLPs) were transfected with the pCAGGS-Env plasmid encoding a human codon optimized CHIKV E3/E2/E1 gene (Salvador et al., 2009). The pHR-CMV-GFP reporter plasmid containing a GFP reporter gene inserted downstream the hCMV immediate early reporter, the HIV-1 packaging signal required for incorporation into VLPs and retroviral LTR sequences necessary for integration in the host cell genome was added to the transfection mixture (Fig. 3A). Control pseudotypes were produced by replacing the CHIKV envelope plasmid by a vector encoding the vesicular stomatitis virus G glycoprotein (VSV-G). Pseudoparticles released in supernatants of transfected cells were of approximately 100–120 nm in size when processed for electron microscopy imaging and contained a conical capsid characteristic of mature HIV-1 structural proteins (Fig. 3B) (Brun et al., 2008). Western blot analysis confirmed that CHIKV envelope was efficiently expressed in producing cells and incorporated into VLPs (Fig. 3C). Moreover, retroviral proteins present at the immature state in cell lysates ($Pr55^{Gag}$) were efficiently cleaved into mature $p24^{CA}$ capsid proteins in cell free samples attesting for the maturation of viral particles. Then, CHIKV- or VSV-G-pseudotyped VLPs normalized according to HIV-1 structural proteins content (50 ng $p24^{CA}$) were incubated with 293T cells in order to determine the functionality of these reagents. As shown in Fig. 3D, both CHIKV- and VSV-G-pseudotyped VLPs directed expression of GFP in the culture, attesting for the capacity of viral glycoproteins to fuse with cells membranes and to deliver a GFP reporter plasmid to the host cell. GFP reporter gene expression was also detected at similar levels in HaCaT cells incubated with normalized amounts of CHIKV envelope-pseudotyped VLPs (Fig. 3D). In these experiments the absence of fluorescence in HaCaT cells incubated with VLPs lacking envelope attested for the absence of pseudo-transduction event (data not shown). Accordingly, membranes of immortalized HaCaT keratinocytes supported efficient fusion with CHIKV envelope glycoproteins.

Analysis of early CHIKV replication events in human keratinocytes

Expression of non-structural proteins nsPs represents an early event in CHIKV life cycle. Once assembled to form the replication complex, these proteins allow replication and intracellular accumulation of the viral genome (Solignat et al., 2009). Thus, early CHIKV replication steps in HaCaT cells were further examined using a CHIKV strain encoding a mCherry fluorescent reporter in frame with the nsP3 nonstructural protein (Kummerer et al., 2012). When used at a MOI of 10 to infect 293T cells, this reporter virus generates a red punctate fluorescence detectable within 9 h of infection (Fig. 4A). The fluorescence persisted and accumulated over time in the culture. Used in similar conditions to infect HaCaT

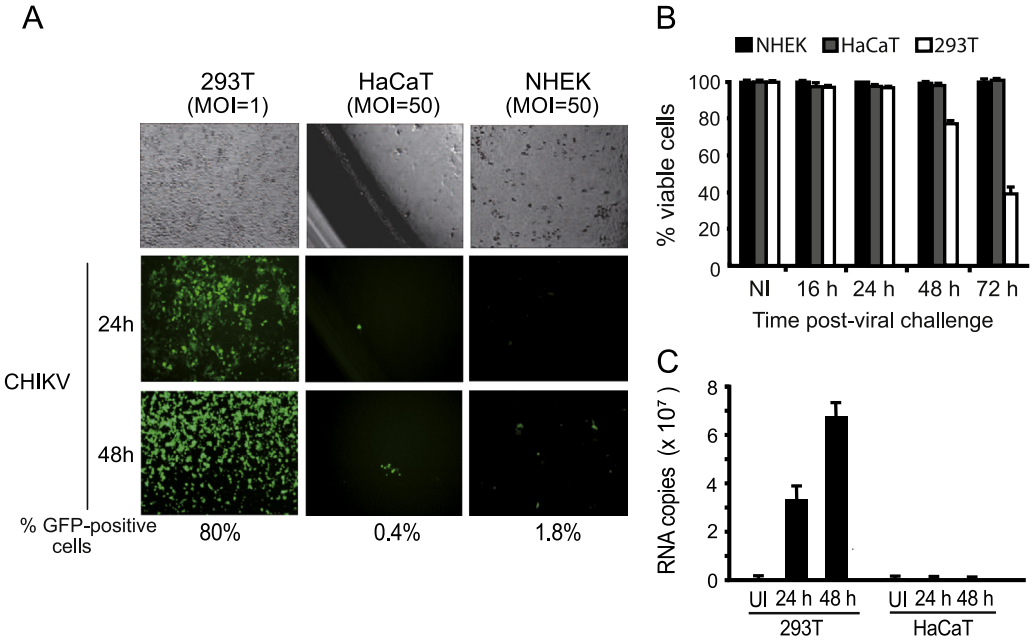

Fig. 1. Human keratinocytes are naturally poorly permissive to CHIKV replication. (A) HaCaT immortalized keratinocytes or neonate primary human keratinocytes (NHEK) were exposed to CHIKV reporter viruses at the indicated MOI. Expression of the GFP reporter gene attesting for viral replication was monitored over time using fluorescence microscopy. Permissive 293T human epithelial cells exposed to a MOI of 1 and then processed in similar conditions are shown as positive control. Percentage of fluorescent cells counted in the culture at 48H post-CHIKV exposure is indicated. (B) CHIKV-exposed HaCaT, NHEK and 293T cells from Fig. 1A were analyzed for cytopathic effects along time using Trypan Blue exclusion assay. Percentages of viable cells in the culture are indicated. (C) Production of progeny virions in culture supernatants from Fig. 1A was monitored by qRT-PCR amplification of CHIKV genomic RNA. CHIKV RNA copy numbers were normalized to GAPDH mRNA copies in the producing cells. Values are the mean of three separate experiments performed in duplicate ± SD.

cells this reporter virus generated very poor fluorescence even when analyzed 48 h post-challenge. When RNA genome synthesis was monitored in the corresponding cultures, qRT-PCR analysis showed a rapid accumulation of CHIKV genomic RNA in 293T cells starting as soon as 6 h post-infection and attesting for the active viral replication (Fig. 4B). To the opposite, RNA copy numbers in HaCaT cells remained stable over time and were below the RNA levels contained in the inoculum (input) attesting for the absence of CHIKV RNA synthesis in these cells.

CHIKV activates an innate immune response in human keratinocytes

Keratinocytes respond to mosquito-borne viruses challenge by mounting an antiviral program characterized by the final upregulation of IFNs genes (Surasombatpattana et al., 2011). This cellular response relies on the transcriptional activation of cytoplasmic RNA helicases retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5), myeloid primary differenciation response 88 (MyD88) as well as toll-like receptor-3 (TLR3) and on upregulation of IFN regulatory factors IRF3 and IRF7 mRNAs. We have therefore examined the transcriptional induction of innate antiviral genes upon CHIKV challenge of this particular cell type. Total RNA prepared from primary human keratinocytes challenged with CHIKV (MOI of 10) for 6 or 24 h was subjected to qRT-PCR analysis using primers for the above mentioned genes. mRNA copy numbers were normalized according to 18S mRNA copies and values were compared with that obtained from cells incubated with supernatant from mock-infected C6/36 cells used to produce CHIKV stocks. As shown in Fig. 5, relative expression of RIG-I (1.6 ± 0.6 folds), MDA-5 (3.9 ± 0.8 folds) and MyD88 (3.2 ± 0.7 folds) were significantly upregulated after 6 h of CHIKV-challenge when compared to mock-challenged cells. The relative expression of TLR3 was not significantly affected. In addition, CHIKV challenge generated a 3 ± 0.8 and a 3.9 ± 1 folds induction of IRF3 and IRF7 mRNAs respectively. Finally, mRNAs of IFN-β and IFNγ were increased by 3.8 ± 1.9 and 6.2 ± 1.5 folds respectively upon CHIKV challenge. Interestingly, transcriptional activation of these genes was not observed after 24 h of infection. Finally, since keratinocytes are cells that both produce and respond to type III interferons (IFN-III) (Odendall et al., 2014), we analyzed the corresponding mRNA levels in response to CHIKV. As shown in Fig. 6A, virus exposure resulted in a significant and continuing increase in IFN-λ mRNA levels starting from 6 h post-exposure and reaching a 800-fold induction after 48 h. Altogether these data indicate that CHIKV induced a significant but transient induction of type I and type II IFN genes in human keratinocytes and results in a sustained type III IFN genes expression in these cells.

CHIKV replication in keratinocytes with perturbation of the IFN signalling pathway

IFNs expression significantly reduces CHIKV replication in IFNs-responsive cell types (Schilte et al., 2010; Sourisseau et al., 2007). We therefore investigated the consequences of IFNs signaling perturbations on CHIKV replication in keratinocytes. First, HaCaT cells were transfected with siRNA targeting either IRF3 or IRF7 transcription factors or with non-targeting siRNA duplexes. After

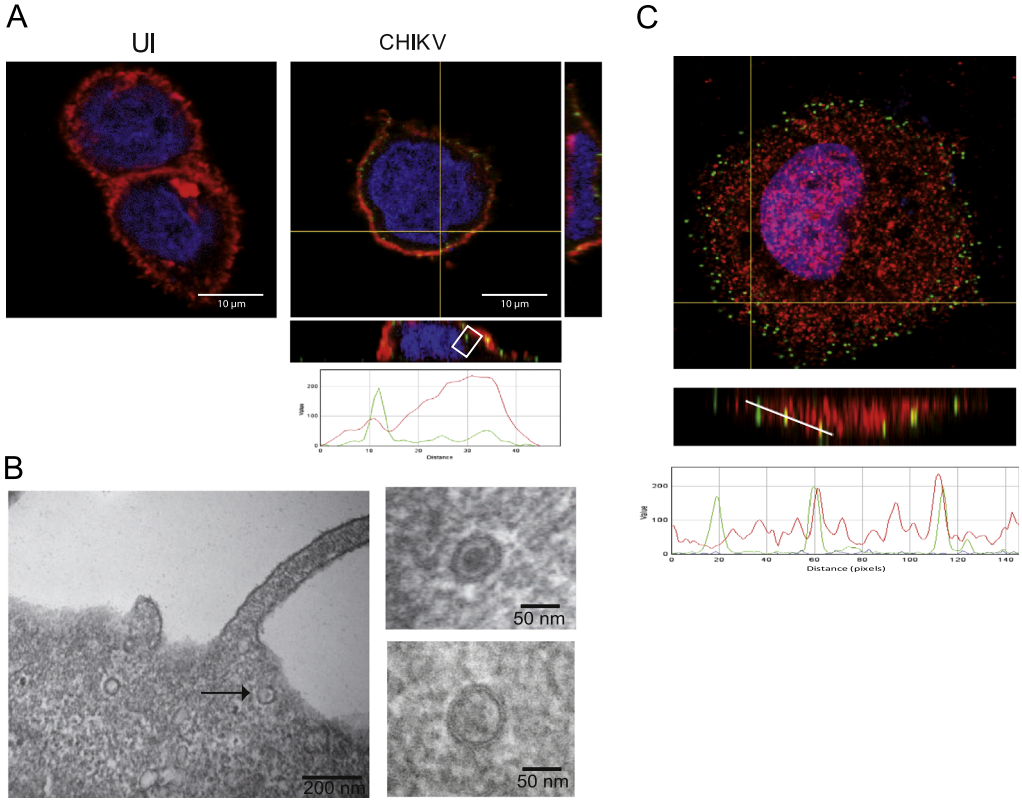

Fig. 2. Viral antigens and assembled particles are detected in the cytoplasm of CHIKV-challenged HaCaT cells. (A) Confocal microscopy analysis of HaCaT cells challenged for 1 h with CHIKV (MOI of 100). Anti-capsid antibodies were revealed with Alexa 488-conjugated secondary antibodies (green), nucleus and cell membranes were labeled with Hoescht dye (blue) and Alexa 647-conjugated WGA (red) respectively. The lateral panels correspond to orthogonal projections of Z-stacks of 12 sequential optical sections spaced 300 nm each, acquired with 100 ms exposure. The white box in the merged image indicates the position of orthogonal analysis. The left panel shows the absence of background signal in uninfected cells (UI). (B) HaCaT cells challenged with CHIKV (MOI = 500) for 10 min were processed for electron microscopy. An arrow indicates the presence of intracytoplasmic CHIKV particle. Enlargements of the cytoplasm containing assembled viral particles are shown. (C) CHIKV capsids (green) and Rab5 endosomal protein (red) were immunostained with specific antibodies. Nuclei were labeled with Hoechst reagent (blue). Orthogonal view showing the colocalization of capsids with Rab5 in high magnification and orthogonal analysis performed at the position indicated by the white line.

72 h in culture, decreased IRF3 and IRF7 expression was evidenced by western blotting analysis of cells lysates (Fig. 7A). Then, the cells were infected with CHIKV reporter viruses. As shown in Fig. 7B, no difference in virus replication was observed between cells transfected with IRF3 or IRF7 targeting siRNAs and the non-targeting control. According to this result, the deregulation of IRF3 or IRF7 genes expression was unable to restore CHIKV replication in HaCaT cells. IRF3 and IRF7 phosphorylation by the TANK-binding kinase (TBK1) and IKKε and subsequent nuclear translocation of these transcription factors are central regulatory steps of the type I interferon-meditated antiviral responses (Sharma et al., 2003). To further evaluate the consequences of IRF3- and IRF7-dependent signaling on the control of CHIKV replication in keratinocytes we took advantage of BX795, a potent inhibitor of TBK1- and IKKε-mediated IRF3 activation (Bain et al., 2007; Clark et al., 2009). HaCaT cells were preincubated with BX795 used at various concentrations before CHIKV challenge. In these conditions, no sign of viral replication could be evidenced from the cultures (Fig. 7C). Finally, the capacity of anti-IFNα/β neutralizing antibodies to control a possible autocrine secretion of type I IFN in the culture was also inefficient at enhancing CHIKV replication (Fig. 7D). In an attempt to counteract any antiviral effects of IFN-λ in the culture we next pre-treated HaCaT cells with AG490, a potent Jak2 inhibitor recently reported to block IFN-λ antiviral effects (Odendall et al., 2014). In our hands, CHIKV replication could not be restored by this inhibitor (Fig. 6B). Accordingly, neither of the strategies used in this study increased CHIKV-replication in human keratinocytes.

Discussion

The present study was conceived to evaluate the behavior of human keratinocytes when challenged with CHIKV. Keratinocytes represent the primary cell population (more than 95%) in the skin (Bos, 2010). This feature has prompted studies aimed at determining the role of this particular cell type as portal of entry for infection of vertebrates by arthropod-borne viruses. In this context, West Nile virus that replicates in the skin of mice infected by blood-suckling mosquitoes was shown to productively infect keratinocytes (Lim et al., 2011). In the recent years, we also reported that Dengue virus, another mosquito-borne pathogen, capable to infect human skin explants (Limon-Flores et al., 2005), is actively replicated in cultures of human keratinocytes (Surasombatpattana et al., 2011). According to these results keratinocytes were proposed as key players of early arboviral infection capable to produce high levels of infectious viruses in the skin favoring viral dissemination to the entire body. Here, we report that CHIKV poorly replicates in cultures of primary and immortalized keratinocytes and despite differentiation status of these cells may be crucial for infection/replication of some viruses (e.g. human papillomavirus (Beglin et al., 2009)), CHIKV behaved independently from the maturation stage of keratinocytes used in this study.

It is well known that *alphaviruses* display a wide spectrum of target cells. Regarding CHIKV, a large variety of in vitro susceptible cells has been identified, including fibroblasts, hepatocytes, neurons, astrocytes, microglial cells, neuroblastoma cells, skeletal muscle progenitor cells, epithelial and endothelial cells (Salvador et al., 2009; Sourisseau et al., 2007; Wikan et al., 2012). In line with this observation, immunohistology analysis detected viral RNA and antigens inside a variety of organs in infected humans and experimentally infected animals, including lymph nodes, spleen, liver, muscle and joints during the acute phase of the infection (Couderc et al., 2008; Her et al., 2010; Labadie et al., 2010; Ozden et al., 2007). Nevertheless, a series of cells can resist CHIKV infection or replication. Immune cells including lymphocytes, dendritic and natural killer cells and well as brain endothelial cells are unable to bind CHIKV envelope glycoproteins and are refractory to infection probably due to the lack of surface receptor(*s*) and/or attachment factor (Salvador et al., 2009; Sourisseau et al., 2007). Although permissive for CHIKV envelope binding and viral fusion, alveolar epithelial cells, muscle fibers, brain-derived microglial cells and human syncytiotrophoblast cells are unable to replicate the virus and to release progeny virions (Solignat et al., 2009; Sourisseau et al., 2007). The data reported herein extend these observations to keratinocytes. Despite

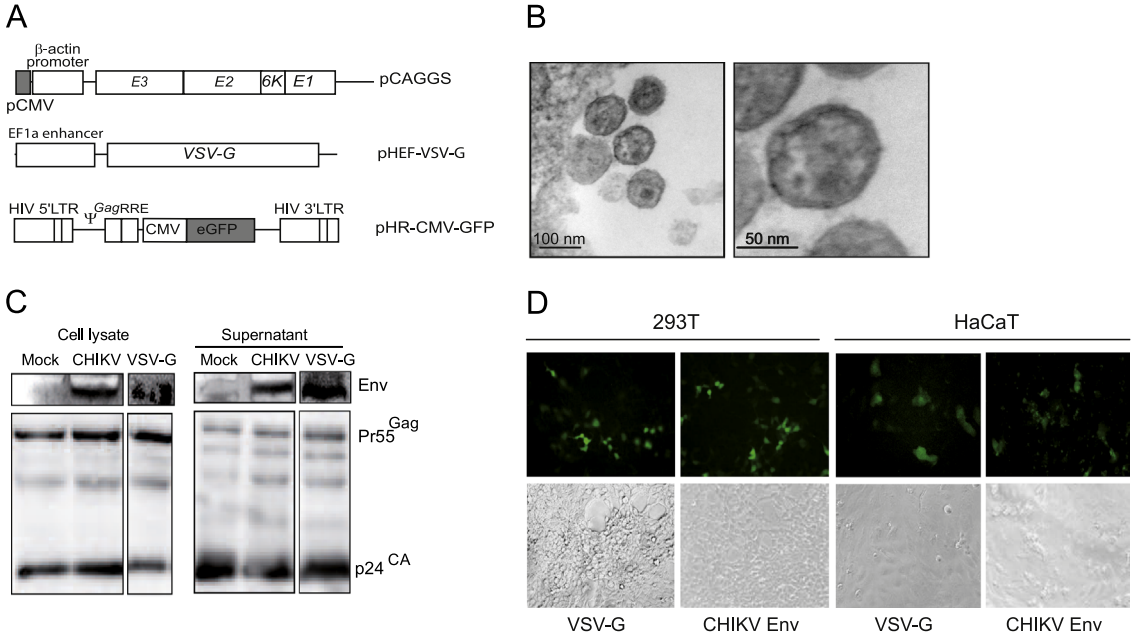

Fig. 3. CHIKV envelope-pseudotyped particles efficiently fuse with HaCaT cells membranes. (A) Organization of plasmids used for production of CHIKV- or VSV-G-pseudotyped retroviral particles. (B) Electron microscopy visualization of CHIKV-pseudotyped particles. (C) Immunoblot detection of envelope glycoproteins (Env) (upper panel) and $Pr55^{Gag}$ and $p24^{CA}$ retroviral structural proteins in VLPs-producing cells and in the corresponding cell free samples. (D) Expression of the pHR-CMV-GFP reported plasmid in 293T or HaCaT cells cultured with VSV-G- or CHIKV-pseudotyped VLPs.

internalizing viral particles that colocalized with Rab5-positive endosomal compartments competent for CHIKV entry in permissive cells (Bernard et al., 2010) and while supporting fusion with viral membranes, keratinocytes are unable to replicate the virus. In these cells, expression of non-structural proteins and synthesis of genomic RNA are impaired. These results produced using *ex vivo* model of infection corroborate histological analysis performed in experimentally infected animals. Indeed, intradermal injection of CHIKV in mouse ears mimicking a mosquito bite evidenced no disseminated viral production at the skin level (Couderc et al., 2008). Viral antigens were instead detected at the level of sebaceous glands, vessels and cartilage and replication was detected in fibroblasts, in the basal layer of the skin (Couderc et al., 2008; Rudd et al., 2012; Schilte et al., 2010). Our observations are therefore in line with the predominant role of fibroblasts proposed during the earliest events in mosquito-to-human transmission of CHIKV.

According to the capacity of IFNs to control CHIKV in mammals (Couderc et al., 2008; Olagnier et al., 2014; Schilte et al., 2010) and in cell cultures (Sourisseau et al., 2007), the stimulation of this signaling axis was considered in our study. Analyzing the intracellular innate immune responses elicited following infection, we found that CHIKV significantly upregulated transcription of a number of cytosolic sensors namely RIG-I, MDA5 and MyD88. These pattern recognition receptors are responsible for sensing the presence of microbial invasion through recognition of RNA species containing 5′ triphosphates and dsRNA fragments (for review see (Jensen and Thomsen, 2012). CHIKV infection also activated expression of IRF3 and IRF7 transcription factors and subsequently stimulated IRF-dependent antiviral genes, including IFN-β and IFN-γ. These data are in line with the early innate immune response against CHIKV elicited in *ex vivo* cultures and at the site of CHIKV inoculation in experimentally infected mice (Schilte et al., 2010; Thangamani et al., 2010; Thon-Hon et al., 2012). Interestingly, we observed that IFN-λ mRNA is highly upregulated in CHIKV-challenged keratinocytes. IFN-λ is part of IFN-III, the most recently described group of small helical cytokines capable of inducing an antiviral state in responsive cells mainly reported as epithelial surfaces and especially keratinocytes (Witte et al., 2009). Its capacity to control replication of viral pathogens, including rotavirus, influenza and metapneumovirus human has been reported (Hermant and Michiels, 2014). Conversely to the regulation of IFN-I and –II genes expression that is relatively well characterized the regulation of IFN-III is less known. However, as IRF-3 and IRF-7 were proposed as common regulators of type I, II and III IFNs induction (Osterlund et al., 2007) strategies targeting these transcription factors were considered in this study. The use of RNA interference strategies and chemical inhibitors validated as perturbators of IRF3- and IRF7-dependent IFN genes expression as well as neutralizing anti-IFNα/β mAbs remained unsuccessful to relieve the intracellular block of CHIKV replication in keratinocytes. However, we cannot rule out that residual IRF3 or IRF7 was sufficient to support antiviral signaling inhibiting CHIKV replication. Moreover, elucidation of events controlling IFN-λ genes activation will provide new tools to decipher the respective contribution of IFN-λ transcriptional activation and of intrinsic replication block in the control of CHIKV replication in keratinocytes. Meanwhile, our study underlines the capacity of keratinocytes to behave as immune sentinels against CHIKV rather than as initial targets for incoming virions and supports that because of its absence of replication in keratinocytes, CHIKV likely behaves differently of DENV and WNV mosquito-borne during early events of vertebrate host colonization.

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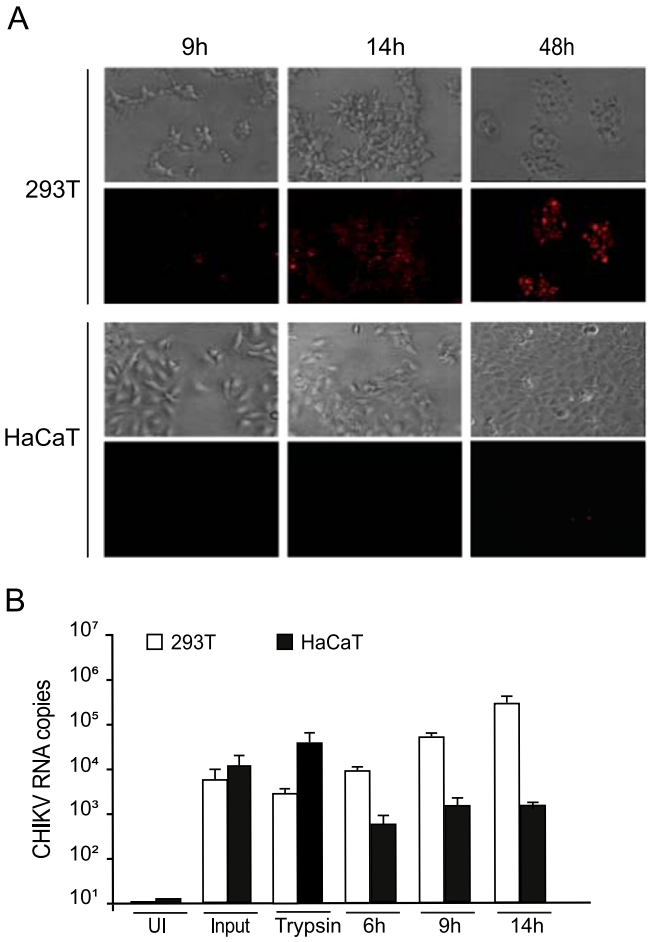

Fig. 4. Expression of CHIKV nonstructural proteins and de novo genomic RNA synthesis are impaired in HaCaT cells. (A) 293T cells or HaCaT cells were challenged with a CHIKV reporter strain expressing the mCherry fluorescent protein in frame with the nsP3 protein. Fluorescence in the cultures was monitored over time using fluorescence microscopy. (B) Viral genomic RNA in the cells was quantified over time using qRT-PCR amplification. Copy numbers were normalized relative to GAPDH mRNA copies in the samples. Control experiments consisted of uninfected cells (UI) and of CHIKV-challenged cells subjected to trypsin-digestion (Trypsin) to remove contaminating RNA contained in membrane-bound particles. RNA copies number contained in the viral input is also indicated (Input). Values represent mean of duplicate experiments ± SD.

Competing interests

The authors have declared that no competing interests exist.

Material and methods

Cells

Aedes albopictus C6/36 cells (ATCC number CCRL-1660) were grown at 28 °C in RPMI medium (Gibco Life Technologies) supplemented with 10% fetal calf serum (Lonza) as previously described (Bernard et al., 2010). Primary human epidermal keratinocytes were obtained from neonatal foreskins (Lonza) and cultured in Keratinocyte Basal Medium-2 supplemented with KGM-2 supplement and growth factors (Lonza). The HaCaT cell line (CLS lines service number

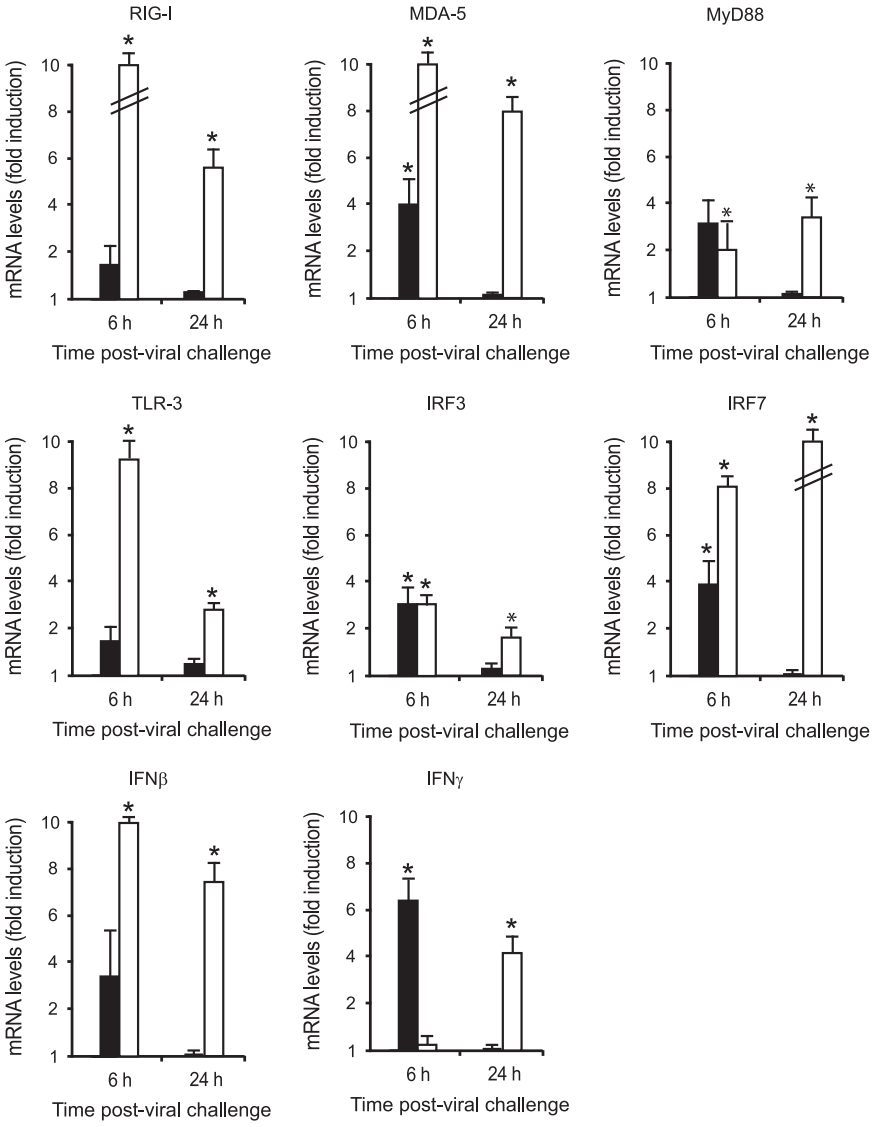

Fig. 5. CHIKV stimulates IFN signaling pathways in primary human keratinocytes. Relative expression of RIG-I, MDA-5, MyD88, TLR-3, IRF3, IRF7, IFN-β and IFN-γ was monitored by real-time quantitative RT-PCR in primary human keratinocytes challenged with CHIKV (MOI of 10) for 6 h and 24 h (black bars) or in cells cultured for the same duration in the presence of poly(I:C) (white bars). RNA copies numbers were normalized according to 18S mRNA levels detected in the same sample. Results are expressed as fold induction relative to mock-infected cells. All experiments were done in triplicates and results are expressed as mean ± standard error. Statistical significance ($p < 0.05$) is indicated by *.

300493) and the HEK293T cell line (ATCC number ACS-4500) were cultured in DMEM medium (Gibco Life Technologies) supplemented with antibiotics and 10% FCS. All human cells were grown at 37 °C in a 5% CO2 atmosphere.

Viruses

CHIKV stocks were produced as follows. The pCHIKV-LR-3′GFP (LR-OPY1-GFP strain) full length CHIKV subgenomic clone expressing GFP at the 5′ end of structural genes (Tsetsarkin et al., 2006) and the CHIKV clone containing the mCherry sequence in frame with nsP3 gene (Kummerer et al., 2012) were transcribed in vitro from the SP6 promoter and the T7 promoter respectively using the mMESSAGE mMACHINE kit (Ambion). RNA (0.5 μg) was then electroporated into 5×10^6 BHK-21 cells with two pulses at 1.5 kV, 25 mF and ∞V. Culture supernatant was harvested at 24 h post transfection and used to infect C6/36 *Aedes albopictus* cells. After two days, supernatant was collected, filtered through 0.45 mm membrane, aliquoted and stored at −80 °C. Viral stocks were tittered using plaque assays as previously reported (Bernard et al., 2010).

Antibodies and reagents

C42 mAbs raised to Semliki Forest nucleocapsid react with the CHIKV capsid protein (Greiser-Wilke et al., 1989). Anti-Rab5 mAbs were from Santa-Cruz. Alexa-Fluor 488-conjugated anti-mouse Ig secondary antibodies and Alexa-Fluor 594-conjugated anti-rabbit Ig were from Life Technologies. Alexa 647-labeled wheat germ agglutinin was purchased from Molecular Probes. Anti-human IFNα/β neutralizing serum (Mogensen et al., 1975) kindly provided by G. Uzé (UMR5235 CNRS) was diluted 1:100 in culture medium 1 h before viral challenge. BX795 and AG490 inhibitors, purchased from Invivogen, were dissolved in DMSO and used as indicated in the text.

Production of CHIKV pseudotypes

Production of CHIKV pseudotypes was achieved through transfection of the 293TSTAR cell line, constitutively expressing HIV-1 *gag* and *pol* genes (Ikeda et al., 2003). Cells were co-transfected with the pCAGGS-EnvCHIKV plasmid encoding codon-optimized

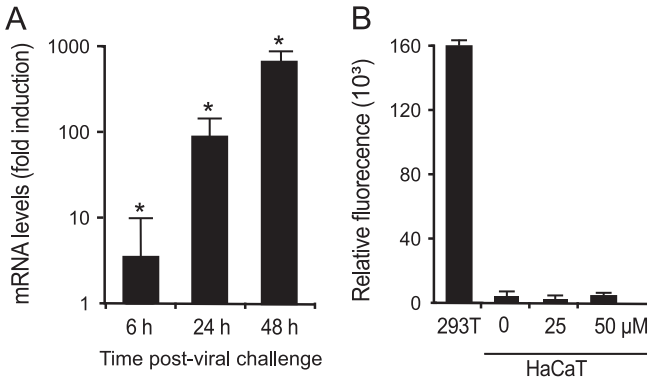

Fig. 6. Type III IFN-λ genes expression and CHIKV replication in keratinocytes. (A) IFN-λ genes expression was monitored over time by real-time quantitative RT-PCR in primary human keratinocytes challenged with CHIKV (MOI of 10). RNA copies numbers were normalized according to 18S mRNA levels detected in the same sample. Results are expressed as fold induction relative to mock-infected cells. Statistical significance ($p < 0.05$) is indicated by *. (B) Replication of the LR-OPY1-GFP viruses in HaCaT cells preincubated with the AG490 Jak2 inhibitor used at the indicated concentrations was evaluated by quantification of the GFP reporter expression. Untreated HaCaT and 293T cells are shown as controls.

CHIKV envelope glycoproteins (Salvador et al., 2009) and with the pHR-CMV-GFP reporter plasmid, (D. Rekosh, University of Virginia), derived from the pHR vector (Naldini et al., 1996) by insertion of a GFP gene in place of luciferase gene. Particles pseudotyped with the vesicular stomatitis virus (VSV) glycoprotein G were produced by co-transfection of the pHR-CMV-GFP with the pHEF-VSV-G plasmid obtained through the AIDS Research and Reference Reagent Program, NIAID, NIH (Chang et al., 1999). The JetPei transfection reagent (PolyPlus Transfection) was used according to manufacturer's recommendations. Forty eight hours post-transfection, viral supernatants were collected, filtered on a 0.45 μm membrane and ultracentrifuged on a 20% sucrose cushion. Viral particles were resuspended in PBS and tittered using the anti-HIV p24 Innotest ELISA assay (Innogenetics). Ectopic expression of proteins in the culture and incorporation into pseudoparticles was analyzed by immunoblot as previously reported (Bernard et al., 2010).

Infection with CHIKV-reporter viruses

Primary or immortalized human keratinocytes were seeded in plates to a confluence of 70-80%. Then, cells were rinsed once with PBS and the CHIKV virus diluted to the desired MOI was added to the cells. The culture was incubated at 37 °C for the duration of the experiment. Viral replication was monitored by visualization of GFP or mCherry expression using fluorescence microscopy (Eclipse TS100, Nikon). For quantitative analysis of fluorescence the cells were lysed with RIPA buffer and fluorescence was measured directly from the cell lysate using an Infinite F200PRO fluorometer (Tecan). Values were normalized according to protein content in the sample determined using the BCA Assay (Pierce).

Quantitative RT-PCR detection of CHIKV RNA

Viral and cellular RNA were extracted with a TRizol-based protocol and tested for mRNA purity and concentration using light absorbance. RNA (0.1 μg) was converted to cDNA with an oligodT (12:18) primer (Invitrogen) and with Superscript III Reverse Transcriptase (Life Technologies) according to manufacturer's instructions. PCR amplification was carried out on $1/10^{th}$ of cDNA in a reaction mix containing 0.4 μM of each primer, and 5 μl SYBR Green master amplification mix (Fast start DNA Master plus SYBR Green I amplification kit, Roche Diagnostics). For each amplification a control reaction was performed in which DNA sample was replaced by water. Reactions were subjected to a first cycle of 10 min at 95 °C followed by 40 amplification cycles of 15 s at 95 °C and 60 s at 60 °C on the RotorGene system (Labgene Scientific). Fluorescence signal was recorded at the end of each cycle. A standard curve was generated from 10^1 to 10^6 copies of pCHIKic-LR-5′GFP plasmid. Primers used for amplification were: 5′–GGCAGTGGTCCCAGATAATTCAAG-3′ and 5′–GCTGTCTAGATCCACCCCATACATG-3′.

Immunofluorescence labeling and confocal microscopy

Cells were grown on glass coverslips to 50–60% confluence and challenged with CHIKV for the indicated time. After extensive washings in PBS, the cells were fixed 20 min at room temperature in 4% formaldehyde (Sigma Aldrich) and permeabilized for 10 min at −20 °C with methanol. After blocking in PBS containing 2% fetal calf serum and 0.1% Tween 20, the cells were incubated overnight at 4 °C with the indicated primary antibodies and for 1 h with the appropriated secondary reagents. Hoechst dye (Sigma-Aldrich) was used to stain the nucleus. After final washes, coverslips were mounted with ProLong Gold anti-fade reagent (Invitrogen). Images were acquired using a Leica SP5-SMD scanning confocal microscope (Leica) equipped with a 63 ×, 1.4 numerical aperture Leica Apochromat oil objective. Confocal Z-slices of 0.3 or 0.5 μm were collected using the LAS AF software (Leica). Orthogonal views were generated by the Image J software and colocalization was analyzed with the Imaris software (Bitplane Scientific Software).

Electron microscopy imaging

Cells were processed for thin-layer electron microscopy as described previously (Brun et al., 2008). Briefly, samples were fixed for 1 h at 4 °C in a solution containing 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4. Cells were then rinsed three times in cacodylate buffer and post-fixed with 1% OsO4. After an additional washing, the cells were incubated for 30 min in 0.5% tannic acid. Dehydratation was obtained with a graded series of ethanol solutions (from 25 to 100%) before embedding in Epok resin at 60 °C for 48 h. Ultra-thin sections were cut on a Reichert OMU2 microtome and then examined under a Hitachi H7100 transmission electron Microscope at 75 kV.

Real-time PCR analysis of innate immune genes expression

Total RNA was extracted from primary keratinocytes using Tri Reagent (Sigma-Aldrich) according to manufacturer's protocol. The RNA was resuspended in 30 μL of RNAse-free distilled water and stored at −80 °C. For each condition, 0.5 μg total RNA was subjected to reverse transcription using M-MLV Reverse Transcription kit (Promega). Real time PCR amplification was run with Roche Light Cycler LC480 device in a 8 μl reaction volume containing 2 μL cDNA, 400 nM of each primer, and 4 μl SYBR Green Master Mix (Roche). The cycling conditions were 45 cycles of 95 °C for 15 s, 60 °C for 15 s and 72 °C for 20 s. mRNA expression (fold induction) was quantified by calculating $2^{-\Delta\Delta CT}$ with 18 s mRNA as an endogenous control. The specific primers used in this study have been previously described (Surasombatpattana et al., 2011). Primers used for IFN-λ are the following 5′- CGCCTTGGAAGAGTCACTCA-3′ and 5′–GAAG CCTCAGGTCCCAATTC-3′.

siRNA transfection

HaCaT cells (12×10^4) were seeded on 24-well plates. The siRNA duplexes specific for IRF3 or IRF7 (Ambion) or non-targeting negative control siRNA (Dharmacon) used at a final concentration of 10 nM was transfected using Lipofectamine 2000 (Life Technologies). After 72 h at 37 °C, the cells were washed

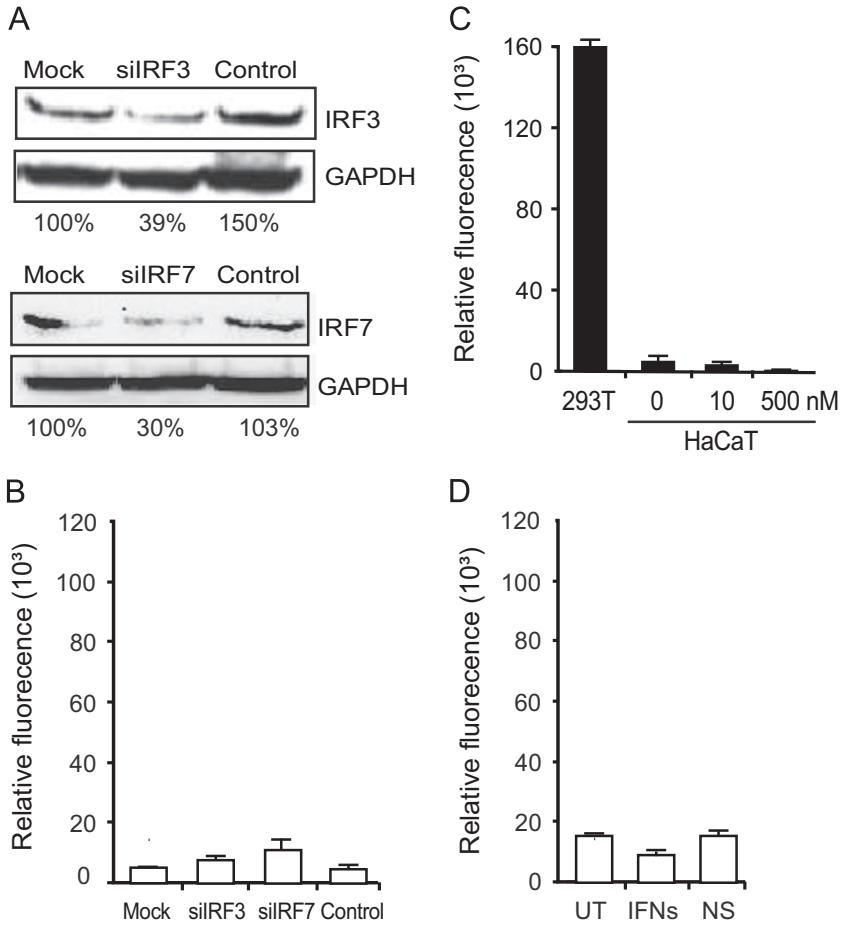

Fig. 7. Consequences of IFN signaling perturbators on CHIKV replication in keratinocytes (A) HaCaT cells were transfected with siRNA to IRF3 or IRF7 or non-targeting duplexes (control). Expression of the corresponding protein was monitored by western blotting after 72 h in culture. Densitometry scanning of western blots in transfected and mock transfected cells (Mock) are indicated. (B) Cells from panel (A) were infected with CHIKV reporter viruses (MOI = 10). Fluorescence intensity was quantified from the total cell lysates. Values are means of duplicates ± SD. (C) Cells preincubated for 6 h with the indicated concentrations of BX795 TBK1 inhibitor were challenged with CHIKV (MOI = 10). GFP expression attesting for CHIKV replication was quantified. Mean values ± SD are indicated. 293T cells and untreated HaCaT cells are shown as controls. (D) HaCaT cells were left untreated (UT) or preincubated for 1 h at 37 °C with anti-IFNα/β (IFNs) or non-specific (NS) antibodies. GFP expression attesting for CHIKV replication was quantified. Mean values ± SD are indicated.

with PBS three times and infected with CHIKV at a MOI of 10. Extinction of IRF3 or IRF7 expression at the time of virus infection was determined by immunoblotting using specific antibodies (Santa Cruz Biotechnologies).

Data analysis and statistical methods

All data are presented as means ± SEM. A comparison of qPCR values was made with the nonparametric Wilcox–Mann–Whitney test. For all experiments, statistical significance was accepted at $p < 0.05$.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.11.013.

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