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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