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## pH-dependent entry of chikungunya virus into *Aedes albopictus* cells

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### ABSTRACT

**Background:** The chikungunya virus (CHIKV) recently caused explosive outbreaks in Indian Ocean islands and India. During these episodes, the virus was mainly spread to humans through the bite of the mosquito *Aedes albopictus*. Concomitantly to the description of symptoms of an unexpected severity in infants and elderly patients, a viral genome microevolution has been highlighted, in particular consisting in the acquisition of an A226V mutation in the gene encoding envelope glycoprotein E1, which was later found to confer an increased fitness for *A. albopictus*. We previously decrypted the entry pathway used by CHIKV to infect human epithelial cells and showed that these mechanisms are modulated by the E1-A226V mutation. In this report we investigated the conditions for CHIKV entry into mosquito cells and we assessed the consequence of E1 gene mutation on these parameters.

**Principal findings:** Our main findings indicate that CHIKV infection of *A. albopictus* cell lines is sensitive to Bafilomycin A1 and chloroquine and to membrane cholesterol depletion.

The E1-226V mutated LR-OPY1 isolate collected during the 2005 outbreak in La Réunion replicated more efficiently than the 37997 African reference strain in C6/36 cells. Moreover, the LR-OPY1 strain displayed greater membrane cholesterol dependence and was more sensitive to inhibition of endosomal pH acidification. Finally, using electron microscopy, we imaged CHIKV entry into C6/36 cells.

**Conclusions:** Our data support that CHIKV is endocytosed into *A. albopictus* cells and requires membrane cholesterol as well as a low-pH environment for entry. These features are modulated in some extent by the A226V mutation in the E1 gene of the LR-OPY1 isolate. Altogether, our data provide information regarding the pathways used by CHIKV to infect *A. albopictus* cells.

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### 1. Introduction

Chikungunya virus (CHIKV) is an *Alphavirus* transmitted to humans by blood-sucking mosquitoes. To ensure its ecological cycle, CHIKV replicates efficiently in both insects and vertebrate hosts. While CHIKV infection is highly cytopathic for mammalian cells (Ozden et al., 2007) and causes an acute febrile illness associated with severe, often debilitating, polyarthralgias in humans (Borgherini et al., 2007; Rulli et al., 2007), *Alphaviruses* replicate in mosquito cells without evident deleterious consequence and results in life-long persistent infection of insect vectors (Karpf and Brown, 1998). Accordingly, CHIKV-host interactions may significantly differ in those two models.

Until recently, CHIKV episodes were restricted to diffuse epidemics in Africa where it was propagated by a variety of

mosquitoes from the *Aedes* family, including *Aedes furcifer*, *Aedes luteocephalus*, *Aedes taylori*, *Aedes africanus* (Diallo et al., 1999; Jupp and Kemp, 1996). Then, the virus was imported in Thailand and India where it has become an urban disease, transmitted largely by *A. aegypti* mosquitoes (for review, see (Chevillon et al., 2008)). During the past decade, important epidemiological outbreaks were reported, first in 2004, when CHIKV re-emerged in Kenya (Sergon et al., 2008) and subsequently in 2005, when the virus spread eastward, causing millions of disease cases throughout countries in and around the Indian Ocean, notably in the island of La Réunion and other neighboring islands of Seychelles, Madagascar, Mauritius and Mayotte (Beltrame et al., 2007; Chastel, 2005). Nowadays, Chikungunya virus continues spreading in India and Southeast Asia countries, including Indonesia, Malaysia, Thailand and Singapore (Pialoux et al., 2007; Ravi, 2006). A highlight of these recent episodes is that the virus is mainly vectored to humans by *Aedes albopictus* mosquitoes. In 2007, a smaller outbreak of Chikungunya disease developed in the Northern Eastern part of Italy, where the local transmission has been made possible by the enormous population of *A. albopictus* and the presence of a viremic patient coming from the Indian Ocean area (Rezza et al., 2007). Importation of CHIKV into Europe, with recent autochthonous cases in France (Gould et al., 2010), indicates that the virus is moving to

**Abbreviations:** CHIKV, chikungunya virus; SINV, sindbis virus; a.a., amino-acid; ECSA, East Central/South Africa.

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novel ecological niches and that Chikungunya infection may become a threat for population of temperate areas.

Molecular epidemiological studies revealed that CHIKV genomes circulating at different time points both in the Indian Ocean and in Asia belong to the same lineage and group of viruses namely the Eastern-Central-South African (ECSA) phylogroup (Schuffenecker et al., 2006). Evolutionary analysis pointed the emergence of a strain having an alanine to valine substitution at codon 226 (A226V) of the envelope 1 (E1) gene in Reunion island (Schuffenecker et al., 2006) and India (Kumar et al., 2008). This mutation was considered as a key evolutionary event that contributed to the transmission and spatial distribution of CHIKV in these regions. Indeed, E1-A226V enhances infectivity of CHIKV for *A. albopictus* and not *A. aegypti* mosquitoes (Tsetsarkin et al., 2007; Vazeille et al., 2007), thereby increasing the transmissibility of CHIKV by *A. albopictus*. Since infectivity of CHIKV pseudotypes was equally enhanced by the presence of this mutation, it was proposed to have a direct effect in the entry process, resulting in differences in cellular tropism (Salvador et al., 2009). These observations have important implications for the design of vector control strategies to fight against the virus in regions at risk of chikungunya fever.

The general picture for the entry of *Alphaviruses* into target cells involves binding of the viral E2 envelope glycoprotein to unknown cell surface receptor(s) and endocytosis of the virus-receptor complex into endosomes (for review see (Strauss and Strauss, 1994)). In endocytic organelles, the low pH environment acts as a cue to induce E1 glycoprotein structural rearrangements, to activate the fusion of the viral and intracytoplasmic host cell membranes and subsequent penetration of the viral genome into target cell cytoplasm (White et al., 1980). We previously evidenced that CHIKV uses a dynamin-dependent endocytic route and requires low endosomal pH and membrane cholesterol for entry into human cells (Bernard et al., 2010). At this time, the cellular pathways hijacked by CHIKV to infect mosquito cells remained largely unravelled. The present study was thus designed to provide some light on CHIKV entry mechanism in vector's cells. To achieve this goal, we took advantage of ultrastructural electron microscopy imaging and of endocytosis perturbators. We also focused on particular aspects that retained attention in the context of mosquito cells infection by *Alphaviruses*, specifically the requirement for low endosomal pH, that was extensively debated in the literature (Hernandez et al., 2001; Hunt et al., 2011; Paredes et al., 2004). This question was addressed through the use of chloroquine, a weak base, and Bafilomycin A1, a v-ATPase inhibitor. Since the E1-226V mutation confers an increased fitness to CHIKV in *A. albopictus* cells, we finally investigated the influence of the recent genome microevolution detected during the Reunion island and India outbreaks on entry routes used by CHIKV (Salvador et al., 2009; Tsetsarkin et al., 2007; Vazeille et al., 2007). We found that exposure to a low-pH environment is required for productive infection of *A. albopictus* cell cultures. Here, we confirm that the E1-A226V mutation present in the LR-OPY1 isolate decreases dependence for membrane cholesterol. Moreover, this mutation was associated with an increased sensitivity to lysomotropic agents. The results accumulated in this study indicate that entry of CHIKV is mediated at least in part, by identical routes in insect and human hosts. These pathways are modulated in some extent by recent genomic microevolution in E1 envelope glycoprotein gene.

## 2. Materials and methods

### 2.1. Cell lines and cultivation

The C6/36 mosquito cell line, derived from *A. albopictus*, was grown at 28 °C and 5% CO<sub>2</sub> in Minimal Essential Medium (MEM) (Sigma) complemented with 10% inactivated FCS and 1% antibiotics.

### 2.2. Production of viral stocks and titration

Full length green fluorescent protein (GFP)-expressing CHIKV subgenomic clones pCHIKic4 (37997 viruses), pCHIK-LRic (LR-OPY1 viruses) and pCHIK-LRic-226A (LR-OPY1<sub>V226A</sub> viruses) were kindly provided by S. Higgs (UTMB, Galveston) (Tsetsarkin et al., 2006, 2007). Each infectious clone was transcribed *in vitro* from the SP6 promoter using the mMMESSAGE mMACHINE kit (Ambion) according to manufacturer's instructions. RNA was then electroporated into BHK-21 cells derived from hamster kidney fibroblasts (ATCC# CCL-10™). Briefly, 5.10<sup>6</sup> cells were washed in ice cold Phosphate Buffer Salin (PBS) and then electroporated with 0.5 µg of RNA with 2 pulses at 1.5 kV, 25 µF and  $\alpha\Omega$ . After two days, cell culture supernatant was harvested, filtered onto 0.22 µm filters, aliquoted and stored at –80 °C. Viral stocks were tittered using plaque assay formation performed on Vero cells, as previously reported (Bernard et al., 2010).

### 2.3. Virus entry assays and drug treatments

Cholesterol depletion was performed by cultivation of the cells for five passages into the appropriate medium supplemented with 10% cholesterol-depleted FCS. Serum depletion in cholesterol was obtained by incubating FCS with 2% CAB-O-Sil (Acros Organics) for 12 h at room temperature as previously described (Weinstein, 1979). Inhibition of endosome acidification was reached through the use of chloroquine and Bafilomycin A1 at concentrations of drugs previously determined to be efficient and without side effect on cell viability. For controls, the cells were treated with the corresponding amount of appropriate dilution solvent. For infection assays, the cells (3.10<sup>5</sup>) were seeded into 24 well plates 24 h before infection. Then, the cells were incubated with drugs as indicated and challenged with CHIKV (m.o.i. of 5) as specified in the Section 3. Infection level was monitored after an additional 16 h in culture, except when specified in the text.

### 2.4. Flow cytometry

Detection of CHIKV infected cells was performed by flow cytometry analysis. Twenty-four hours after the viral challenge, the cells were extensively washed, trypsinized and fixed with a 4% paraformaldehyde solution for 20 min at 4 °C. After additional washings, the cells were resuspended in PBS and GFP expression was monitored directly by flow cytometry. Fluorescence intensity was recorded (20,000 events) on a COULTER EPICS XL flow cytometer (Beckman Coulter). Student *t*-tests, calculated using the VassarStats Website (<http://faculty.vassar.edu/lowry/VassarStats.html>), were used to determine differences between infection levels and were.

### 2.5. Electron microscopy

Cells were allowed to bind viruses for 30 min at 4 °C, and then shifted to 28 °C for 10 min to promote viral entry. Then, the cells were processed for thin-layer electron microscopy as described previously (Brun et al., 2008). Briefly, the cells were fixed for 1 h at 4 °C in a solution containing 2.5% glutaraldehyde in 0.1 M cacodylate pH 7.4. The cells were then rinsed three times in cacodylate buffer and post-fixed with 1% OsO<sub>4</sub>. After an additional washing, the cells were incubated for 30 min in 0.5% tannic acid. Dehydration was obtained with a graded series of ethanol solutions (from 25 to 100%) before embedding in Epok resin at 60 °C for 48 h. Ultrathin sections were cut on a Reichert OMU2 microtome and then examined under a Hitachi H7100 transmission electron microscope.

### 3. Results

#### 3.1. Ultrastructural study of CHIKV infection in mosquito cells

We first investigated the general pathways used by CHIKV to enter mosquito cells by performing ultrastructural electron microscopy imaging of infected cells. C6/36 cells challenged with CHIKV (37997 strain) for a short period of time (10 min at 28 °C) were processed for electron microscopy. Virions at different stages of the entry process could be observed. Spherical viral particles in the size range of 50–70 nm surrounded by a bilayer were found near or docked at the plasma membrane. Some particles were observed embedded within electron-dense invaginations of the plasma membrane. At these sites, morphology of the plasma membrane evoked in some cases the biogenesis of clathrin-coated pits (Fig. 1A and B). However, some observations indicated the presence of invaginations that could coincide with the formation of uncoated pits (Fig. 1C). Overall, these pictures agree with a model of CHIKV entry into mosquito cells through endocytosis. Within the cytoplasm, viral particles were frequently observed within single cytoplasmic vesicles or accumulated inside large vacuoles surrounded by membrane bilayer (Fig. 1D and E). In some cases, vesicles containing single virus particles were observed fusing with larger intracytoplasmic vacuoles resulting in the formation of large electro-lucent intracellular compartments where viral particles accumulate (Fig. 1F). These data suggest that in mosquito cells, CHIKV particles may undergo a multistep vesicular transport pathway from the plasma membrane to a deeper intracytoplasmic compartment.

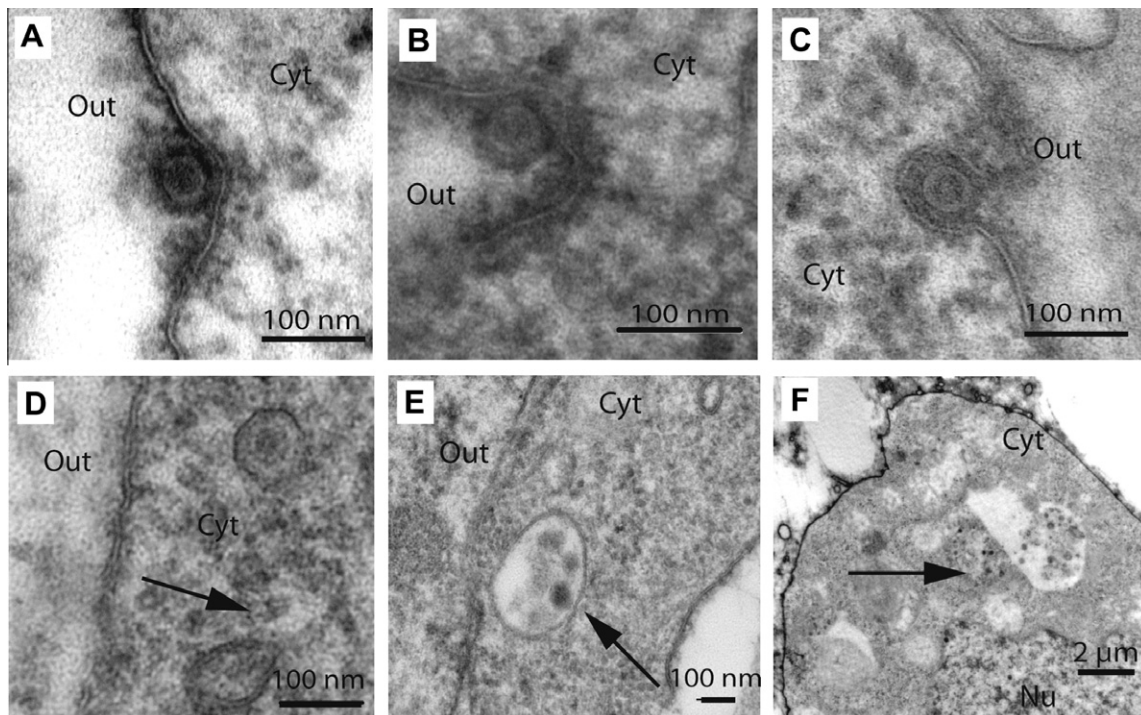
#### 3.2. CHIKV requires membrane cholesterol for infection of *A. Albopictus* cell lines

Entry of *Alphaviruses* in mosquito cells as well as infection of human cells by CHIKV are dependent upon membrane cholesterol

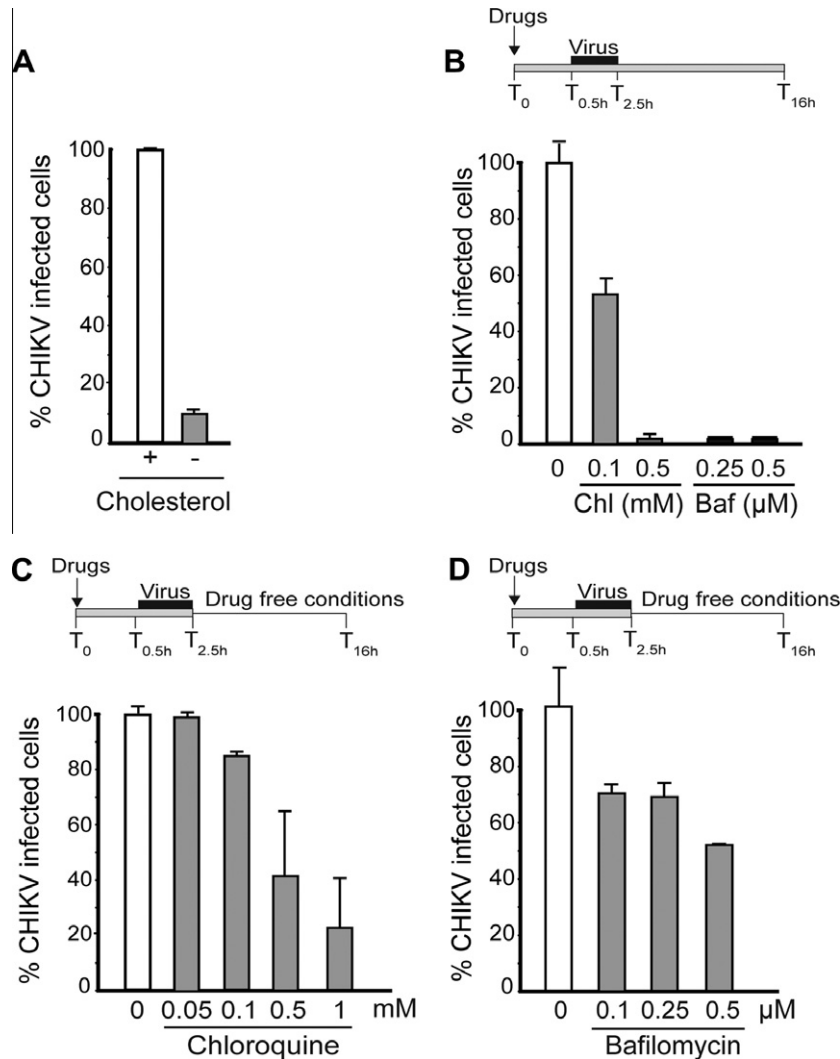
content (Bernard et al., 2010; Chazal and Gerlier, 2003). Here, we reinvestigated the sensitivity of mosquito cells infection to membrane cholesterol depletion. Insects cannot synthesize cholesterol *de novo* and depend on dietary cholesterol for their physiological requirements (Krebs and Lan, 2003). In culture, they incorporate cholesterol from serum and can thus be depleted to significant levels by successive passages in medium supplemented with low-cholesterol containing serum, without deleterious effects or compensatory changes in lipid composition. Accordingly, C6/36 cells were delipidated by serial passage in medium containing CAB-o-Sil-treated serum as described in the Section 2. This approach clears over 96% of lipids present in serum and impairs entry of cholesterol-dependent viruses (Weinstein, 1979). Cells were then challenged with an infectious clone of CHIKV derived from the 37997 African reference strains expressing a GFP reporter protein. After extensive washing, the infection was allowed to proceed for 16 h before expression of GFP was determined by flow cytometry as an indicator of successful virus infection. As shown in Fig. 2A, cultures of C6/36 cells maintained in the presence of delipidated serum were less susceptible to CHIKV infection than cells cultured in standard conditions. This result is consistent with the expected effects of cholesterol depletion of the cells (Schuffenecker et al., 2006; Tsetsarkin et al., 2007; Vashishtha et al., 1998).

#### 3.3. Effects of lysomotropic agents on CHIKV infection of *A. Albopictus* cell lines

*Alphaviruses* are internalized from the cell surface by receptor-mediated endocytosis and reaches an endosomal compartment where the fusion occurs. It is generally accepted that once internalized, acidification of the endosomal vacuole generates conformational changes in *Alphaviruses* envelope glycoproteins, allowing the fusion of viral and cell membranes and subsequent RNA release into the cell (Kielian et al., 2010). Accordingly, we explored whether CHIKV can infect mosquito cells maintained in the



**Fig. 1.** Electron microscopy imaging of CHIKV entry into mosquito cells. C6/36 mosquito cells were challenged with the 37997 CHIKV strain. After 10 min in culture, the cells were harvested and processed for electron microscopy as describe in the Section 2. Cyt: cytoplasm, Out: extracellular space. CHIKV particles in the intracellular space were docked to the plasma membrane near clathrin-coated (A and B) or uncoated (C) structures. Accumulation of virions in intracytoplasmic vacuoles of various sizes was observed (D, E and F). Viral particles are pointed by an arrow and the scale is indicated by the bar. Nu: nucleus, Cyt: cytoplasm, Out: extracellular space.



**Fig. 2.** Requirement for membrane cholesterol and low endocytic pH for CHIKV infection of C6/36 cells. (A) C6/36 cells cultured in complete medium (+) or maintained in the presence of delipidated serum (–) were challenged with CHIKV (37997 strain, m.o.i = 5). Infection was monitored by flow cytometry analysis of intracellular GFP expression. Values are a mean of two separate experiments performed in quadruplicate + SD. (B, C and D) Infection of C6/36 cells with CHIKV was performed in the presence of Bafilomycin A1 or chloroquine. (B) Increasing concentrations of drugs were added to the cells 30 min before the viral challenge and maintained throughout the experiments. (C) and (D) C6/36 cells were prepared and infected as in (B). Two hours after virus exposure, the cells were extensively washed and cultured in drug free conditions for the rest of the experiment. Level of CHIKV-positive cells was monitored by flow cytometry analysis of intracellular GFP expression. Values are a mean of two separate experiments performed in triplicate + SD.

presence of the vacuolar type-H<sup>+</sup>-ATPase inhibitor Bafilomycin A1 or cultured with chloroquine, a weak base widely used to inhibit endosomes acidification. The concentrations of drugs used were chosen based on earlier studies showing that they were efficient at inhibiting vacuolar pH acidification in mosquito cells and efficiently inhibit low pH-dependent entry of viruses into this cell type (Acosta et al., 2008; Chu et al., 2006; Colpitts et al., 2007; Hunt et al., 2011; Yoshimori et al., 1991). C6/36 preincubated with increasing concentrations of drugs for 30 min were challenged with CHIKV. The drugs were maintained in the culture during the viral challenge and throughout the experiment until flow cytometry analysis of intracellular viral antigens (Fig. 2B). In our experimental conditions, 60% of untreated cultures became infected attesting for non saturating conditions of infection (data not shown). For each condition, the drug treatment was controlled to have no deleterious effect on culture viability (data not shown). Using this approach, increasing concentrations of Bafilomycin A1 or chloroquine, significantly reduced the percentage of CHIKV-positive cells in the culture when compared with cultures of infected

cells maintained in the presence of corresponding concentrations of drug solvent (Fig. 2B). To avoid any effect of drugs on intracellular steps of viral replication, infection assays were repeated with cells maintained with drugs for 30 min before and during 2 h of viral challenge. Then the cells were washed and the infection was allowed to proceed in drug free medium. In these experimental conditions, levels of CHIKV-positive cells decreased with increasing concentrations of Bafilomycin A1 and chloroquine (Fig. 2C and D). Accordingly, CHIKV infection of mosquito cells requires endosomal pH acidification.

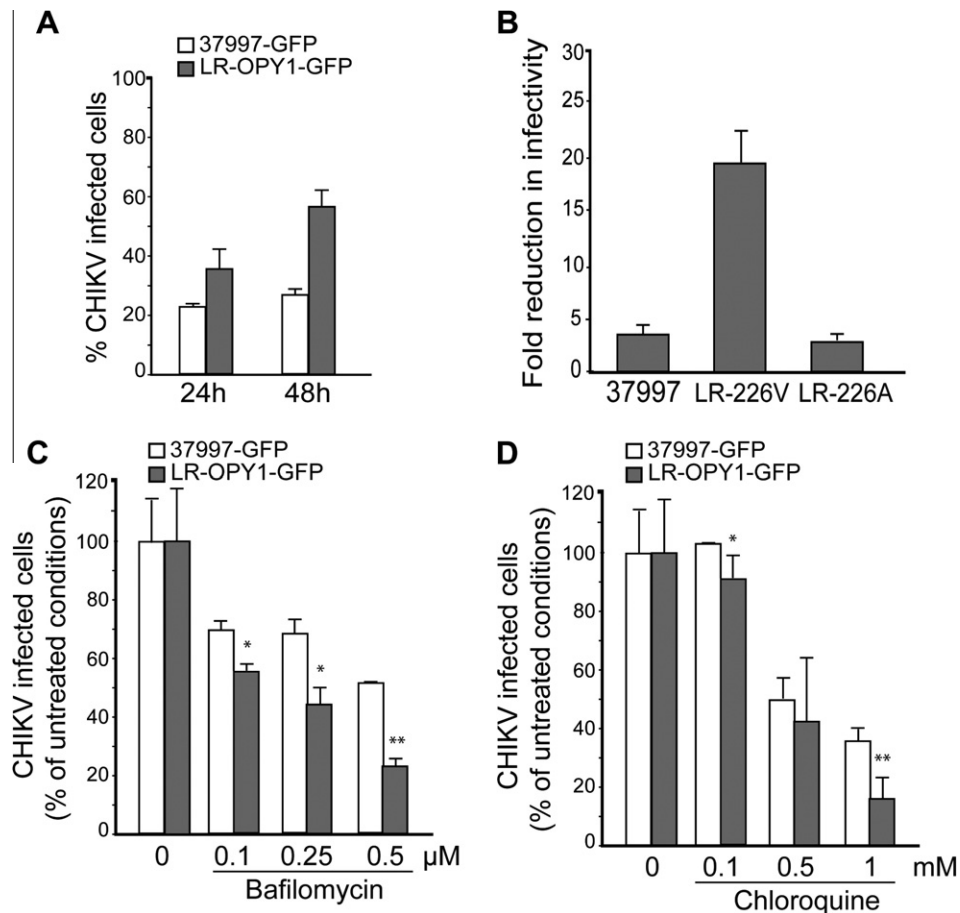
Of note, the percentage of CHIKV-positive cells measured when the cells were maintained in drug free conditions after viral challenge were higher than those observed when Bafilomycin A1 or chloroquine were present throughout the experiment. Accordingly, both drugs, not only inhibit viral entry, but also likely interfere with a post-entry step of CHIKV replication. For chloroquine, this effect is consistent with the capacity of the drug to perturb intracellular steps of replication of a variety of viruses (Savarino et al., 2003).

### 3.4. Comparative study of entry pathways used by African and Indian Ocean prototype CHIKV strains to infect *A. albopictus* cells

The LR-OPY1 strain circulating during the 2005–2006 Indian Ocean episode and the 37997 strain have been grouped into the East/Central/South African phylogroup and into the group of West African strains of CHIKV, respectively. The two strains share 85% nucleotide sequence identity. Specifically, they differ regarding the nature of amino acid at position 226 in E1 (Schuffenecker et al., 2006). Significant differences have been reported regarding the competence of *A. albopictus* mosquitoes for infection and replication of both strains and acquisition of the single A226V mutation by the LR-OPY1 strain was proposed to coincide with an increased fitness for *A. albopictus* (Tsetsarkin et al., 2007; Vazeille et al., 2007). Here, we performed parallel infection of C6/36 cells exposed to normalized amounts (according to p.f.u.) of 37997 or LR-OPY1 viruses to evaluate their respective behavior. Levels of GFP expression were monitored at different time post infection and we observed that the replication of LR-OPY1 viruses was higher by 2-fold that of 37997 viruses (Fig. 3A). Accordingly, the LR-OPY1 may have gained a selective advantage for replication in this particular cell type as previously proposed (Tsetsarkin et al., 2007). In order to evaluate the influence of the E1-226V mutation on entry pathway used by CHIKV, delipidated C6/36 cells were challenged with normalized amounts of the 37997 or LR-OPY1 viruses

in conditions described above. The requirement for membrane cholesterol was estimated as the ratio of infected cells quantified after delipidation of cell membranes to that measured in standard culture conditions. Infection of the C6/36 cell line by the 37997 strain was less significantly impaired by cholesterol depletion than observed for LR-OPY1 viruses (Fig. 3B). The direct implication of residue E1-226 in sensibility of CHIKV to cholesterol depletion was further confirmed by using a LR-OPY1<sub>V226A</sub> mutant derived from the LR-OPY1 strain and bearing a reverse valine to alanine substitution at position 226. The reversal mutation decreased sensitivity of the LR-OPY1 to cholesterol depletion to levels observed for the 37997 strain. According to these data, E1-A226 residue is determinant for cholesterol dependence in mosquito cells.

We recently found that 37997 and LR-OPY1 strains differ in some extent regarding their sensibility to lysomotropic agents during infection of mammalian cells (Bernard et al., 2010). Here, we compared the requirement of both strains in low endosomal pH for infection of C6/36 cells. Increasing concentrations of Bafilomycin A1 or chloroquine were added to C6/36 cells. After 30 min in culture, the cells were challenged with CHIKV in the presence of drugs. After extensive washings, the infection was allowed to proceed for 16 h in culture before flow cytometry detection of CHIKV-positive cells. In these conditions, infection levels in cells challenged with the LR-OPY1 strain were repeatedly lower than in cells exposed to 37997 viruses. Except when 0.5 mM



**Fig. 3.** Comparative analysis of 37997 and LR-OPY1 CHIKV for cholesterol and low endosomal pH requirement. (A) Replication efficiency of normalized dose of 37997 and LR-OPY1 in C6/36 cells (m.o.i = 5) was determined by flow cytometry analysis of GFP-positive cells after 24 or 48 h in culture. (B) C6/36 cells cultured in standard conditions or delipidated as described in the Section 2 were challenged with 37997, LR-OPY1 (LR-226V) or LR-OPY1<sub>V226A</sub> (LR-226A) viruses. Infectivity was determined as the ratio of CHIKV-infected cells in standard condition, determined by flow cytometry, to that of CHIKV-infected cells in delipidated cell cultures. Values are a mean of two separate experiments performed in quadruplicate + SD. (C) Susceptibility of 37997 and LR-OPY1 to endocytic pH inhibitors was determined in infection experiments performed in the presence of increasing concentrations of Bafilomycin A1 (C) or chloroquine (D). Infection level was monitored by flow cytometry analysis of intracellular GFP expression. Values are a mean of two separate experiments performed in quadruplicate + SD. Statistical significance is indicated (\* $p < 0.05$ ; \*\* $p < 0.01$ ).

chloroquine was added to the culture medium, differences observed for the two viruses were statistically significant as ascertained with Student *t*-tests (Fig. 3C and D). Accordingly, the LR-OPY1 isolate was slightly more sensitive to Bafilomycin A1 and to chloroquine than the 37997 strain (Fig. 3C and D). Altogether, these data support that re-emerging CHIKV displays an increased requirement for membrane cholesterol and for low endocytic pH.

#### 4. Discussion

In the present study, we determined the requirements of Chikungunya virus entry in mosquito cells. We specially considered the C6/36 cell line derived from *A. albopictus*, the predominant vector involved in dissemination of the virus during recent outbreaks. We used electron microscopy imaging and chemical inhibitors to evaluate the functional requirement of key players suspected to participate in viral entry. We report that CHIKV requires both membrane cholesterol and vacuolar pH acidification to infect vector's cells. Moreover, electron microscopy imaging supports that the virus penetrates into target cells through endocytosis. The most commonly accepted mechanism of entry for *Alphaviruses* is via endocytosis and the subsequent acidification of the vesicle leading to membrane fusion (for review see (Kielian et al., 2010)). Structural rearrangement generated by low endosomal is generally accepted as a prerequisite for fusion of viral envelope with cellular membranes during early steps of Alphavirus infection (Kielian et al., 2010). This model was established mainly using mammalian models of *in vitro* infection and extended to mosquito cells for some viral models (Colpitts et al., 2007; Kielian et al., 2010; Phalen and Kielian, 1991). In contrast with this dogma, a number of observations provided support for a pH-independent entry route in mosquito cells. Sindbis virus (SINV), the *Alphavirus* prototype, was repeatedly reported to retain the capacity to infect mosquito cells maintained in the presence of lysomotrophic drugs, thereby supporting the contribution of a pH-independent route in this particular cell type (for review see (Kononchik et al., 2011)). Our results indicate that CHIKV infection of C6/36 cells is sensitive to inhibitors of the v-ATPase and chloroquine, a weak base that accumulates inside the acidic parts of the cell and inhibits acidification of endocytic compartments. Recently, we have reported the first characterization of the endocytic routes used by CHIKV to infect human epithelial cells (Bernard et al., 2010). We found that CHIKV uses an Eps15-dependent endocytosis entry pathway, and requires membrane cholesterol, functional early endosomes and acidic pH conditions for productive infection. A similar requirement for low endosomal pH was also reported for infection of muscular cells (Salvador et al., 2009). Accordingly, the present study supports that a conserved mechanism is required for entry into insect, human cells. Nevertheless, we found that concentrations of acidic pH inhibitors effective in reducing CHIKV infection of *A. albopictus* cells were significantly higher than in human cells. Therefore, sensitivity to these drugs could vary according to the host species. Interestingly, the electron microscopy analysis of CHIKV infected cultures performed in complement of the inhibitor-based study revealed the presence of CHIKV particles docked to *bona fide* clathrin-coated and uncoated vesicles. These patterns indicate that different endocytic pathways could be hijacked by CHIKV. The use of specific endocytic perturbators will be of help for future characterization of the nature of endocytic vesicles supporting CHIKV entry and fusion. Interestingly, in some cases, CHIKV particles directly connected at the plasma membrane were observed (B. Gay unpublished observations). In these rare cases, a direct connection between the virus and the cell cytoplasm could be visualized. These pictures were reminiscent of “pore”-like structures that were proposed to mediate the direct penetration of

SINV genome at the cell plasma membrane through a “pore” formed following binding of the virus to its receptor (Kononchik et al., 2011). Nevertheless, since most viral particles were associated with diverse intracytoplasmic vacuoles and docked to invaginations of the plasma membrane, this case was marginal and in our hands endocytosis accounted predominately for CHIKV penetration through endocytosis as suggested for related viruses (Strauss and Strauss, 1994).

Along the objective of this work, was the comparative analysis of entry routes used in mosquito cells according to the nature of amino-acid residue in E1 envelope glycoprotein. Indeed, the reemerging CHIKV collected in La Reunion during the 2005–2006 outbreak characterized by the E1-A226V mutation, displays an increased fitness for *A. albopictus* as reported herein. The underlying mechanisms remained to be elucidated. Infection and membrane fusion of Semliki Forest virus and Sindbis virus with mammalian cells have been shown to be heavily stimulated *in vitro* and *in vivo* by the presence of cholesterol and sphingolipids in the target membrane (Lu et al., 1999; Phalen and Kielian, 1991). Such requirement is related to the capacity of the E1 envelope ectodomain of *Alphaviruses* to associate with cholesterol-enriched membrane domains (Ahn et al., 2002; Chazal and Gerlier, 2003). The comparative analysis of the 37997 strain with the LR-OPY1 isolate and its reverse LR-OPY1<sub>V226A</sub> mutant confirmed that in C6/36 cells, CHIKV requirement for membrane cholesterol was related to the nature of residue at position 226 in E1. This result agrees with previous reports (Tsetsarkin et al., 2007). The use of lysomotrophic agents revealed that the 37997 African reference strain and the LR-OPY1 virus both require endosomal vacuoles acidification for optimal infection of *A. albopictus* cells. However, LR-OPY1 appeared slightly more sensitive to inhibition of low endosomal pH. This result is reminiscent of the differential sensitivity to lysomotrophic agents and weak bases reported for both viruses using human epithelial cells (Bernard et al., 2010; Salvador et al., 2009; Sourisseau et al., 2007). Overall, the LR-OPY1 and the 37997 strains of CHIKV displayed subtle different requirement for viral entry in *A. albopictus* cells. The impact of such differences on virus fitness remains to be investigated in depth. Considering different virus family, the fusion reaction of viral and cellular membranes required for penetration of the capsids into the cell cytoplasm can take place either in early or late endosomes. Both the lipids composition and the intravacuolar pH slightly vary according to the maturation state of the endosomal vacuole (Kielian et al., 2010). We have previously reported that functional Rab5-positive early endosomes are required for CHIKV infection of human cells (Bernard et al., 2010). Since the re-emerging LR-OPY1 strain displayed some particular features regarding entry with a higher dependence upon membrane cholesterol and an increased requirement for low endosomal pH, the impact of the E1-226V mutation on the vesicular compartment used for fusion needs to be investigated.

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#### Competing interests

The authors have declared that no competing interests exist.

## Author contributions

Conceived and designed the experiments: LB. Performed the experiments: EB, BG, MS. Analyzed the data LB, NC, BG. Wrote the paper LB, NC, CD.

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