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Rôle du cholestérol, de la protéine SAMHD1 et de la salive d'*Aedes aegypti* dans l'infection des cellules cutanées par le virus Chikungunya

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ABSTRACT

Chikungunya virus (CHIKV) is a re-emerging mosquito-borne alphavirus that has been spread worldwide. The dissemination of this virus is a threat to human health since there is no approved vaccine or appropriate antiviral agents to control viral infection. The global expansion of the virus is preceded by biting of infected *Aedes* mosquitos, which injects saliva containing the virus into the skin of the human host. Searching for effective antiviral compounds and understanding of the molecular mechanisms involved in host-virus or vector-virus-host interactions are crucial for controlling viral spread.

Using different molecular and cellular strategies, we demonstrated that the FDA approved drug, imipramine, which has the capability to disturb intracellular cholesterol transport inhibits CHIKV replication in human skin fibroblasts. Imipramine was found to affect both the fusion and replication steps of the viral life cycle. Moreover, it also strongly inhibited the replication of several *Flaviviridae* family members, including Zika, West Nile and Dengue virus. We have also determined the global proteomic profile of Chikungunya and Zika virus infected human skin fibroblasts, and found that several interferon-stimulated proteins and antiviral response proteins are significantly up-regulated in the infected cells. More importantly, our results also provided for the first time a role of SAMHD1 in arbovirus infection of human skin fibroblasts. Finally, we demonstrated that *Aedes aegypti* saliva enhances CHIKV replication in human skin fibroblasts. To our knowledge, this is the first report showing the importance of *Aedes aegypti* saliva on promoting CHIKV infection via down regulation of the genes involving type I IFN secretion in the infected human cutaneous cells.

Keywords: Arbovirus, Chikungunya virus, Zika virus, Human skin fibroblasts, Imipramine, SAMHD1, *Aedes aegypti*, Saliva

RÉSUMÉ

Le virus Chikungunya (CHIKV), arbovirus en pleine ré-émergence, a envahi rapidement de nombreuses zones géographiques du monde. La propagation mondiale de ce virus constitue une menace pour la santé humaine car il n'y a pas de vaccin ou d'agents antiviraux appropriés pour contrôler l'infection virale. La transmission du virus s'effectue lors de la piqure d'un moustique infecté du genre *Aedes*, qui injecte sa salive contenant le virus dans la peau de l'hôte humain. Afin de contrôler la dissémination du virus, il est primordial de développer des recherches sur l'identification de molécules antivirales et de comprendre les mécanismes moléculaires impliqués dans les interactions hôte-virus et/ou vecteur-virus-hôte.

En utilisant différentes stratégies moléculaires et cellulaires, nous avons étudié le potentiel antiviral de l'Imipramine, une molécule déjà commercialisée et qui a la capacité de perturber le transport du cholestérol intracellulaire. Nous avons démontré que cette molécule est capable d'inhiber la réplication du CHIKV dans les fibroblastes cutanés humains. Nous avons mis en évidence que l'Imipramine affectait à la fois les étapes de fusion et de réplication pendant le cycle de réplication du virus. En outre, la molécule a également fortement inhibé la réplication de plusieurs *Flavivirus* comme le virus Zika (ZIKV), le virus du Nil occidental et le virus de la Dengue. Nous avons également déterminé le profil protéomique global des fibroblastes humains infectés par le CHIKV ou le ZIKV. Cela nous a permis de mettre en évidence les modulations significatives de plusieurs protéines stimulées par l'interféron et de protéines impliquées dans à la défense anti-virale dans les cellules infectées. Plus important encore, nos résultats montrent pour la première fois le rôle de la protéine SAMHD1 dans l'infection des fibroblastes cutanés par les arbovirus.

Enfin, compte tenu des fortes interactions entre l'hôte, le vecteur et le CHIKV, l'effet de la salive du moustique *Ae. Aegypti* sur l'infection virale a été étudié. À notre connaissance, cette étude est la première à montrer l'importance de la salive d'*Ae. aegypti* sur la facilitation de l'infection du CHIKV, dans des fibroblastes cutanés, à travers la régulation des gènes impliqués dans la réponse interféron de type I.

Mots-clefs : Arbovirus, virus Chikungunya, virus Zika, fibroblastes cutanés humain, Imipramine, SAMHD1, *Aedes aegypti,* salive

TABLE OF CONTENTS

ABSTRACT 2

LIST OF ABBREVIATIONS 5

LIST OF FIGURES 7

LIST OF TABLES 8

CHAPTER I: GENERAL INTRODUCTION 9

1. ARBOVIRUSES 10

1.1 General information 10
1.2 Alphaviruses 11
1.3 Flaviviruses 12
1.4 Geographic distribution 13
1.5 Transmission Cycle 15
1.6 Type I IFN induction 17
1.7 Clinical manifestations and chronic sequelae 19
1.8 Laboratory Diagnosis and Detection 20
1.9 Dengue, Chikungunya and Zika virus co-infection in humans 20
1.10 Disease prevention and control 22

2. CHIKUNGUNYA VIRUS 24

2.1 Classification 24
2.2 Geographic distributions and Outbreaks 25
2.3 Viral structure 26
2.3.1 Structural proteins 26
2.3.2 Non-structural proteins 26
2.4 Viral Replication 27
2.4.1 Receptor-mediated endocytosis 27
2.4.2 Life cycle 27
2.5 Mechanisms of immune evasion 28

3. INTRACELLULAR CHOLESTEROL TRAFFICKING 30

3.1 Cholesterol and viral entry 30
3.2 Cholesterol and viral replication 31
3.3 Cholesterol and viral morphogenesis 32
3.4 Targeting cholesterol as an antiviral strategy 32

4. SAMHD1 protein 33

4.1 SAMHD1 structure and function 33
4.2 SAMHD1 Regulation 35
4.3 Role of SAMHD1 in the innate immunity 36

5. MOSQUITO SALIVA 37

5.1 Saliva and host haemostasis 38
5.2 Saliva in the modulation of the host immune system 39
5.3 Role of mosquito saliva on arboviruses infection 40

6. RESEARCH OBJECTIVES.....42

CHAPTER II: 43

IMIPRAMINE INHIBITS CHIKUNGUNYA VIRUS REPLICATION IN HUMAN SKIN FIBROBLASTS THROUGH INTERFERENCE WITH INTRACELLULAR CHOLESTEROL TRAFFICKING

CHAPTER III:.....73

SAMHD1 ENHANCES CHIKUNGUNYA AND ZIKA VIRUS REPLICATION IN HUMAN SKIN FIBROBLASTS

CHAPTER IV:.....111

***Aedes Aegypti* SALIVA ENHANCES CHIKUNGUNYA VIRUS REPLICATION IN HUMAN SKIN FIBROBLASTS**

CHAPTER V: 123

CONCLUSIONS AND PERSPECTIVES

CHAPTER VI:.....132

Résumé des travaux en français

REFERENCES 145

APPENDICES.....156
AWARDS

Le Meilleur poster: *de la Journées de l'Infectiopôle Sud 2016*

La médaille de bronze: *de la Journées de l'Infectiopôle Sud 2015*

LIST OF ABBREVIATIONS

Ae.	*Aedes*
AGS	Aicardi-Goutières syndrom
APCs	Antigen presenting cells
BFV	Barmah Forest virus
CCR	Chemokine receptor
CHIKV	Chikungunya virus
DENV	Dengue virus
ER	Endoplasmic Reticulum
GAGs	Glycosaminoglycans
HIV	Human immunodeficiency virus
IFNs	Interferons
IFIT	Interferon-induced proteins with tetratricopeptide repeats
IFITM	Interferon-induced transmembrane protein
IL	Interleukin
IRF	Interferon Regulatory Factor
ISFV	Insect-specific flaviviruses
ISG	Interferon Stimulated Genes
ISRE	Interferon stimulated response elements
JAK-STAT	Janus kinase1 activates signal transducer and activator of transcription1
JEV	Japanese Encephalitis Virus
MBFV	Mosquito-borne group
MDA5	Melanoma Differentiation-Associated protein 5
MyD88	Myeloid Differentiation primary response gene (88)
MVEV	Murray Valley encephalitis virus
NKFV	No known vector viruses
NLR	Nucleotide oligomerization domain (Nod)-like receptors
NLS	Nuclear localization signal
NS1	Non-structural glycoprotein-1
ONNV	O'nyong'nyong virus
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PI4P	Phosphatidylinositol 4-Phosphate

LIST OF ABBREVIATIONS (Continued)

PKR	Protein kinase R
RC	Replication complex
RLR	retinoic-acid inducible gene-I-like receptors
PRRs	Pattern-Recognition Receptors
RIG-I	Retinoic-acid inducible gene-I
RNA	Ribonucleic acid
RRV	Ross River virus
SAMHD1	Sterile Alpha Motif and Histidine-aspartic Domain containing protein 1
SARS	Severe acute respiratory syndrome
SGE	Salivary gland extract
SFV	Semliki Forest virus
SINV	Sindbis Virus
SLEV	St Louis encephalitis virus
TBEV	Tick-borne encephalitis virus
TGN	Trans-Golgi network
TLR	Toll-like receptors
TRIF	TIR-domain-containing adapter-inducing interferon-β
Tyk2	Tyrosine kinase 2
V/W/E-EEV	Venezuelan, Western, and Eastern equine encephalitis viruses
WNV	West Nile virus
YFV	Yellow fever virus
ZIKV	Zika virus

LIST OF FIGURES

Figure 1. Alphavirus phylogenetic tree..... 11

Figure 2. Flavivirus genus classification. 12

Figure 3. Global distribution of some of the most important arboviruses. 14

Figure 4. Arbovirus transmission cycles..... 16

Figure 5. Innate immune signaling via type I IFNs. 18

Figure 6. Global distribution of DENV, ZIKV and CHIKV. 20

Figure 7. Chikungunya Phylogenetic tree. 24

Figure 8. Geographic distribution of CHIKV and its primary vectors. 25

Figure 9. Alphavirus genome structure. 26

Figure 10. Alphavirus replication cycle. 28

Figure 11. Mechanism of immune invasion by Chikungunya virus.. 29

Figure 12. Cholesterol and replication organelles. 31

Figure 13. SAMHD1 structure and its function domain. 34

Figure 14. Essential Role involved by SAMHD1. 34

Figure 15. Global distribution of *Ae. aegypti* and *Ae. albopictus* and its morphology..... 37

LIST OF TABLES

Table 1. List of the most important arboviruses and their characteristics. …… 10

Table 2. Clinical comparison of Dengue, Chikungunya and Zika. …… 19

CHAPTER I:
GENERAL INTRODUCTION

1. ARBOVIRUSES

1.1 General information

Arbovirus (Arthropod-Borne virus) is a term used to refer to a group of viruses that are transmitted to vertebrate hosts by hematophagous (blood feeding) arthropod vectors such as mosquitoes (*Culicidae*), sand flies (*Psychodidae*), biting midges (*Ceratopogonidae*), black flies (*Simuliidae*) and ticks (*Ixodidae* and *Argasidae*) (Gubler, 2002). Arboviruses have been causing human treat disease for at least a thousand years including: alphaviruses (*Togaviridae*), flaviviruses (*Flaviviridae*), bunyaviruses (*Bunyaviridae*) and some viruses in the families *Reoviridae* and *Rhabdoviridae* (Weaver and Reisen, 2010). Arboviruses are taxonomically diverse, belonging to eight viral families and 14 genera. Although, there are currently 534 viruses listed in the International Catalogue of Arboviruses, 134 of the 534 viruses have been reported to cause illness in humans (Rehle, 1989). The most public health importance belongs to arboviruses and their characteristics are summarized in Table 1.

Table 1. List of the most important arboviruses and their characteristics.
Adapted from (Hadinegoro et al., 2015, Iranpour et al., 2016, Mayer et al., 2017)

Family (Genus)	Virus	Vector	Vertebrate host	Diseases in humans	Geographic distribution	Vaccine
Togaviridae	Chikungunya	M	Humans, Primates, Birds, Cattle, Rodents	SFI	Africa, Asia, Europe, Americas, Oceania	NO
(Alphaviruses)	Ross river	M	Humans, Marsupials	SFI	Australia, South Pacific	NO
	Mayaro	M	Primates, Other mammals, Birds	SFI	South and central America	NO
	O'nyong-nyong	M	?	SFI	Africa	NO
	Sindbis	M	Birds	SFI	Asia, Africa, Europe, Americas	NO
	Barmah forest	M	Birds? Marsupials, Others?	SFI	Oceania	NO
	Eastern equine encephalitis	M	Birds, Horses, Other mammals	SFI, ME	Americas	YES
	Western equine encephalitis	M	Birds, Horses, Rabbits	SFI, ME	Americas	NO
	Venezuelan equine encephalitis	M	Horses, Rodents, Other mammals, Birds	SFI, ME	Americas	YES
Flaviviridae	Dengue 1-4	M	Humans, Primates	SFI, HF	Asia, Americas, Africa, Europe, Oceania	YES
(Flaviviruses)	Zika	M	Humans, Primates	SFI	Asia, Africa, Central and South America, Oceania	NO
	Yellow fever	M	Humans, Primates	SFI, HF	Africa, South America	YES
	Japanese encephalitis	M	Birds, Pigs	FSI, ME	Asia, Pacific, Australia	YES
	Murray valley encephalitis	M	Birds	SFI, ME	Australia	NO
	Rocio encephalitis	M	Birds	SFI, ME	South America	NO
	St. Louis encephalitis	M	Birds	SFI, ME	Americas	NO
	West Nile	M	Birds, Horses, Other mammals	SFI, ME	Asia, Americas, Africa, Europe, Oceania	NO
	Kyasanur forest disease	T	Primates, Rodents, Camels	SFI, HF, ME	India, Saudi Arabia	YES
	Omsk haemorrhagic fever	T	Rodents	SFI, HF	Asia	NO
	Tick-borne encephalitis	T	Birds, Rodents	SFI, ME	Europe, Asia, North America	YES
Bunyaviridae	Sandfly fever	S	?	SFI	Europe, Africa, Asia	NO
(Bunyaviruses)	Rift valley fever	M	?	SFI, HF, ME	Africa, Middle East	YES
	La Crosse encephalitis	M	Rodents	SFI, ME	North America	NO
	California encephalitis	M	Rodents	SFI, ME	North America, Europe, Asia	NO
	Congo-Crim. haemorrhagic encephalitis	T	Rodents	SFI, HF	Europe	YES
	Oropouche fever	Mi	?	SFI	Central and South America	NO
Reoviridae	Colorado tick fever virus	T	Rodents	SFI	North America	NO

HF, haemorrhagic fever; ME, meningoencephalitis; SFI, systematic febrile illness; Mi, Midges; M, Mosquitoes; S, Sandflies; T, Ticks.

1.2 Alphaviruses

The family *Togaviridae* is comprised of two genera; alphavirus and rubivirus which contains about 31 viruses that divided into eight phylogenetic groupings (Weaver and Rice, 2000). Although, the organization of their genomes is similar, the phylogenetic analysis shows that alphaviruses and rubiviruses are quite divergent (Koonin and Dolja, 1993). The alphaviruses are widely distributed throughout the world, inhabiting all of the continents except Antarctica. In humans, symptoms of alphaviral infections range from fever, rash, nausea and polyarthritis to fatal encephalitis. Whilst mortality is low for many alphaviruses, associated disease can be debilitating, with clinical sequelae lasting from months to years in some patients (Weaver and Lecuit, 2015). Until now, there are currently no effective vaccines or treatments for human alphavirus infections.

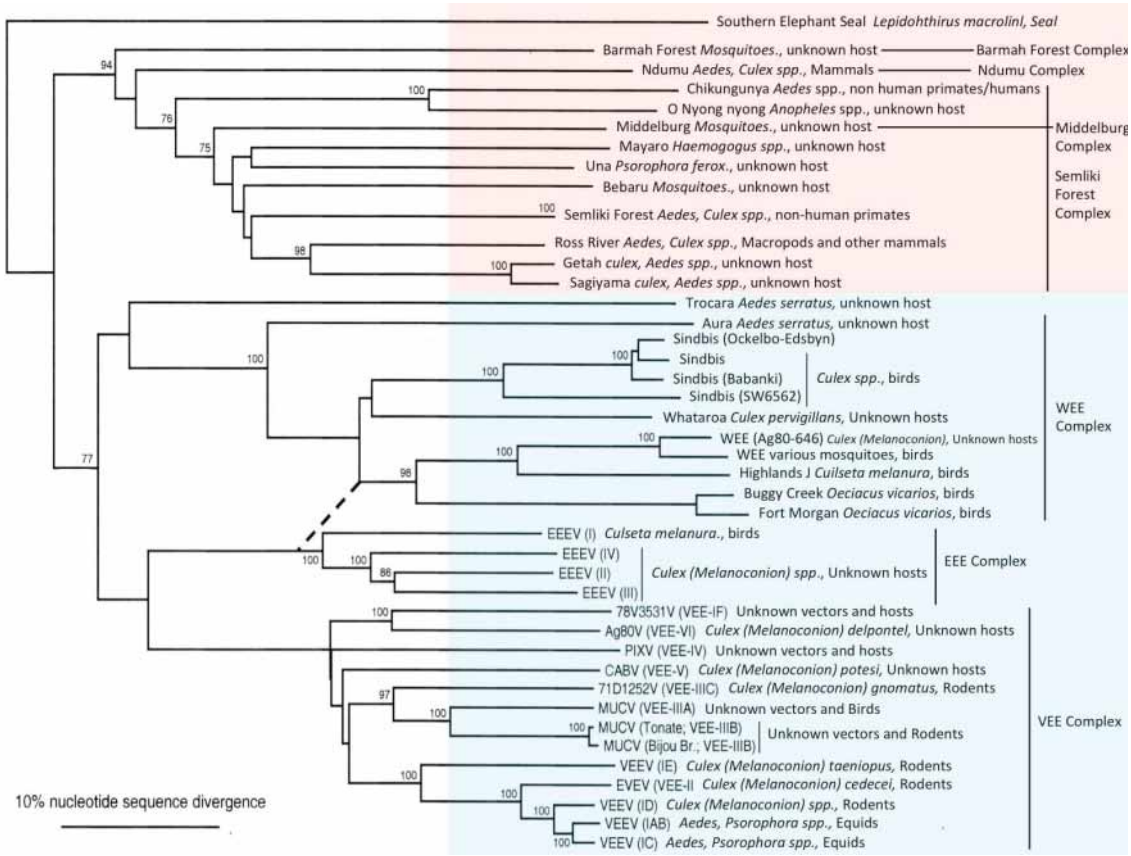

Figure 1. Alphavirus phylogenetic tree. The tree generated from full-genome alignment of both ORFs, excluding portions of the nsP3 and capsid made using Bayesian methods and rooting. The orange shading indicates viruses classified as Old World alphaviruses, while blue shading indicates those classified as New World viruses. Modified from (Forrester et al., 2012, Powers et al., 2001).

Alphaviruses are classified phenotypically and phylogenetically into two groups; the Old World viruses and New World viruses (Figure 1). The Old World alphaviruses include the Sindbis virus (SINV) group, Barmah Forest virus (BFV), O'nyong'nyong virus (ONNV), Ross River virus (RRV), Semliki Forest virus (SFV), and Chikungunya virus (CHIKV). While, the New World alphaviruses include Venezuelan, Western, and Eastern equine encephalitis viruses (V/W/E-EEV) and are mostly associated with encephalitic disease in horses and humans. Aquatic alphaviruses have been isolated from lice, but evidence that they transmit these viruses is lacking (Forrester et al., 2012, Powers et al., 2001). The arthropod vectors, including numerous mosquito species as well as potentially mites, and vertebrate hosts of alphaviruses are also diverse.

1.3 Flaviviruses

Flaviviridae family gathers a large variety of viruses that share morphological and genomical organizations as well as replicative strategies, but differ considerably in their biological and antigenic properties. The family includes three genera; Pestivirus (from Latin *Pestis*, or plague), Hepacivirus (from Greek *Hepar*, or liver) and Flavivirus (from Latin *Flavis*, or yellow) (Calisher et al., 1989).

Initially, flaviviruses were considered as a group within the *Togaviridae* family. However, the distinctive morphological, replicative and biochemical characteristics of flaviviruses led to define the *Flaviviridae* family in 1985, which was initially composed of the single genus Flavivirus (Westaway et al., 1985). Flaviviruses are divided into four large groups: the mosquito-borne group (MBFV), the tick-borne group (TBFV), the vertebrate-specific flaviviruses group, referred to 'no known vector viruses' (NKFV), and the ones that have been only isolated from insects, which constitutes a growing group of viruses known as insect-specific flaviviruses (ISFV) (Figure 2) (Blitvich and Firth, 2015).

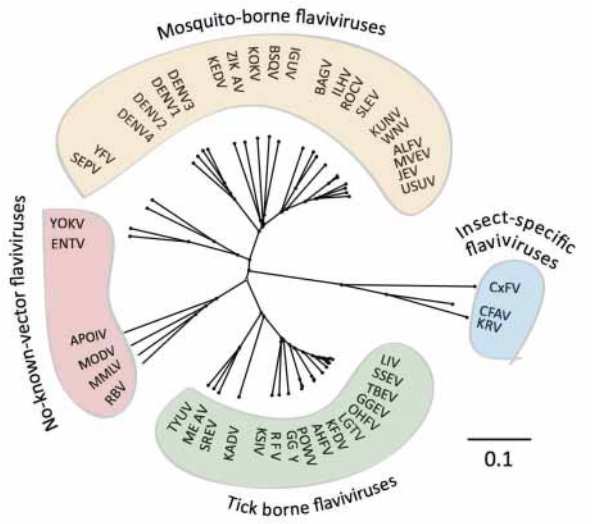

Figure 2. Flavivirus genus classification. (Blitvich and Firth, 2015)

Mosquito-borne flaviviruses include Dengue virus (DENV), Yellow fever virus (YFV), West Nile virus (WNV) and Zika virus (ZIKV), all of which are human pathogens of global concerns (Vasilakis and Weaver, 2016). Other MBFV localized public health concerns include St Louis encephalitis virus (SLEV) and Murray Valley encephalitis virus (MVEV).

Tick-borne encephalitis flaviviruses are essentially found in the Northern hemisphere of Europe and Asia. Most of them cause neurological diseases in humans and can be classified according to their hosts. For example, TBEV, Langat, Powassan and Omsk hemorrhagic fever viruses are transmitted by ticks form the Ixodes genus to mammalian hosts, especially rodents. Others like Gadgets Gulley and Tyuleniy viruses have been isolated from seabirds-associated ticks from the Ornithodorus and Ixodes genera (Gaunt et al., 2001).

NKFV members have never been isolated from mosquitoes and do not seem to replicate in mosquito cell lines. Their hosts are mammals (rodents and bats) and some of them, such as Rio Bravo Virus, are zoonotic and can infect humans (Volkova et al., 2012).

ISFV can be divided into two distinct phylogenetic groups. The first group currently consists of approximately 12 viruses including cell fusing agent virus, Kamiti River virus and Culex flavivirus. These viruses are phylogenetically distinct from all other known flaviviruses. The second group currently consists of nine viruses and includes Chaoyang virus, Nounané virus and Lammi virus. ISFV have been shown to enhance or suppress the replication of medically important flaviviruses in co-infected mosquito cells (Blitvich and Firth, 2015). WNV was significantly higher transmitted when co-inoculated Honduras *Cx. quinquefasciatus* compared to mosquitoes inoculated with WNV alone. While, the suppression of the replication of WNV, JEV and SLEV were observed in the co-inoculation of mosquito (C6/36) cells with Nhumirim virus (Kenney et al., 2014).

1.4 Geographic distribution

In the past 20 years, global epidemic arboviral activity has dramatically increased. The outbreaks of DENV, ZIKV and CHIKV infection in the Caribbean over the past decade have demonstrated the potential of these arboviruses to pose a global public health (Cao-Lormeau, 2016). Arboviruses are transmitted in zoonotic cycles involving non-human primates and arboreal mosquitoes, and have entered human-to-human cycles involving urban *Ae. aegypti* and *Ae. Albopictus* transmission, except those of WNV which is transmitted mainly by genus *Culex* (Hubálek and Halouzka, 1999).

In recent years, the prevalence of vector-borne diseases has expanded considerably, due to intensification of human travel and transcontinental commerce. The number of cases

has increased in endemic regions and the cases have also spread into new regions where the viruses never existed before (Mayer et al., 2017).

When we observe the geographical distribution of DENV, ZIKV, and CHIKV over the past decade, DENV expansion appears a continuous process. However, the emergence of CHIKV and ZIKV has dramatically expanded over the time (Figure 3).

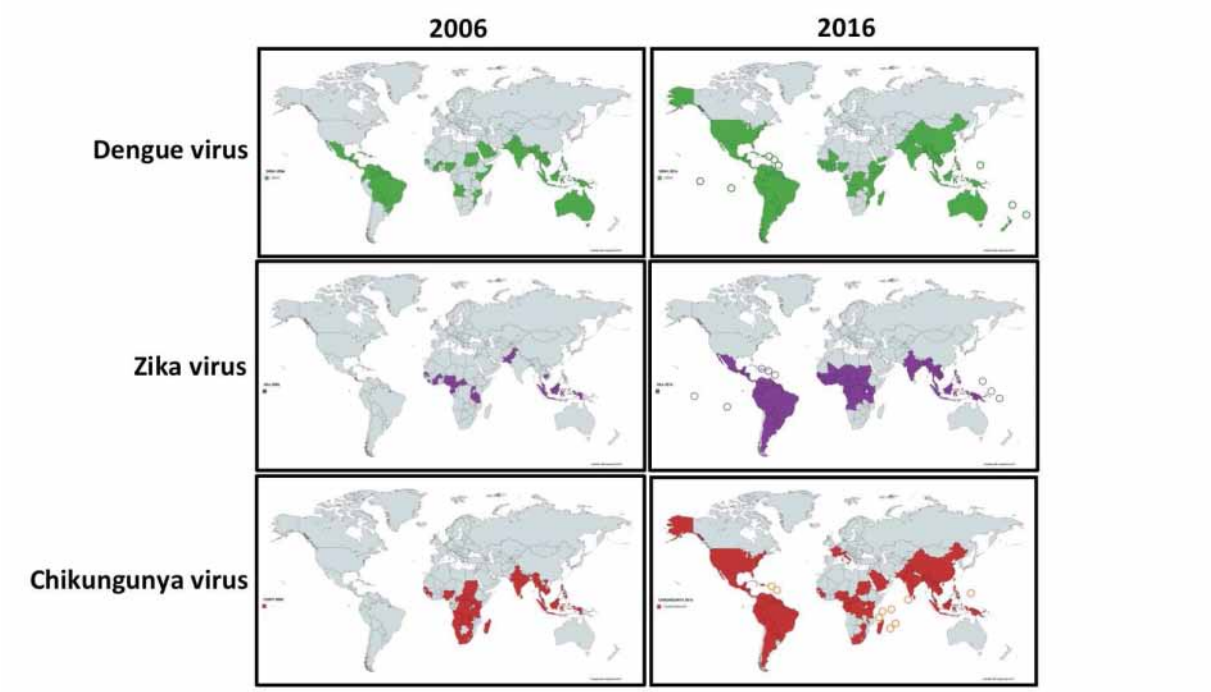

Figure 3. Global distribution of some of the most important arboviruses. Adapted from (Cao-Lormeau, 2016, CDC, 2016, 2017b, Kindhauser et al., 2016, WHO, 2017)

DENV are considered the most important arboviruses in the world, now endemic in more than 100 countries in Africa, the Americas, the Eastern Mediterranean, Southeast Asia and the Western Pacific (Guzman and Harris, 2015). The incidence of DENV has increased dramatically in recent years with over 2.5 billion people now at risk of contracting DENV. It has been estimated that the annual number of DENV infections could be from 50 to 400 million cases with 25,000 deaths reported annually (Mustafa et al., 2015).

ZIKV was first isolated from a human in Uganda in 1952, then sporadic cases of human infection were detected in Africa and later in Southeast Asia (Hamel et al., 2016). In 2007, ZIKV emerged for the first time outside Africa and Asia, in Yap State in Micronesia. Six years later, the virus caused a large outbreak in French Polynesia and then spread to other Pacific islands (Musso et al., 2015). In May 2015, autochthonous cases of ZIKV infection were confirmed in Brazil. By the end of the year, Brazil had declared an outbreak, and the virus had spread to several neighboring countries (http://www. paho.org). In February 2016,

25 countries and territories from the Americas, Africa and Asia have reported ZIKV circulation and an additional 7 countries have reported ongoing outbreaks of ZIKV infection.

Countries reporting ongoing outbreaks are the Bolivarian Republic of Venezuela, Brazil, Cabo Verde, Colombia, El Salvador, Martinique and Panama (http://www.who.int).

CHIKV was isolated in 1952-1953 following a outbreak in Tanzania (ROBINSON, 1955). Then, the first marked outbreak was reported in La Réunion Island during 2005-2006, associated with severe clinical presentations including the first confirmed deaths from CHIKV (Weaver and Lecuit, 2015). During the next 10 years, CHIKV caused several outbreaks in the Indian subcontinent, Asia, and Central Africa, and autochthonous transmission was reported in Europe, in Italy and France (Grandadam et al., 2011, Watson, 2007). In the western hemisphere CHIKV was initially identified on Saint Martin Island in October, 2013, and then the virus rapidly spread to countries and territories in the Americas. From the onset of the outbreak to early August, 2016, autochthonous transmission of the virus has been confirmed in 48 countries or territories in the Caribbean, Central America, South America, and North America, with more than 1 million suspected cases (Burt et al., 2017).

1.5 Transmission Cycle

Most arboviruses are zoonotic and have a maintenance enzootic cycle involving the arthropod vectors (mosquitoes, flies, ticks, etc.) and vertebrate hosts (birds, mammals, rodents, etc) as reservoir hosts. The enzootic cycles of some viruses, for example VEEV and YFV occur in sylvatic habitats, whereas the enzootic cycle of WNV can also occur in urban habitats. Enzootic and bridge vectors might be involved in transmission. Some arboviruses such as VEEV and Japanese encephalitis virus (JEV) achieve further amplification and human disease by exploiting domestic animals with a rural epizootic cycle, such as equines and pigs, increasing spillover and causing epidemics in rural settings. Epizoonic rural cycle of arboviruses do not usually involve humans, however, they can be infected (spill over) and import the disease to urban areas and cause focal epidemics in the presence of competent vectors.

DENV, ZIKV and CHIKV transmit among enzootic sylvatic and epidemic urban cycle (Figure 4A). Such viruses have adapted to urban cycles in which humans are the main reservoir. In case of WNV, humans produce viral titers not high enough to transmission that are considered as‘‘dead-end’’ hosts (Figure 4B).

Even if mosquito bites are the main mode of transmission, some cases of non-vector-borne infection have also been reported. Transfusion transmission of DENV in Hongkong, Singapore, Brazil Puerto Rico and Australia and WNV in the Americas has been demonstrated (Anez et al., 2012). Perinatal transmission, probably following viral crossing of the placenta or during the delivery by viraemic mothers, and sexual transmission were reported in case of ZIKV (Hamel et al., 2016). Recently, Evans-Gilbert reported that two severe cases of CHIKV vertically transmitted neonatal died within 2 days after the presentation of abdominal distension, reduced perfusion pressure, and hypotension (Evans-Gilbert, 2017).

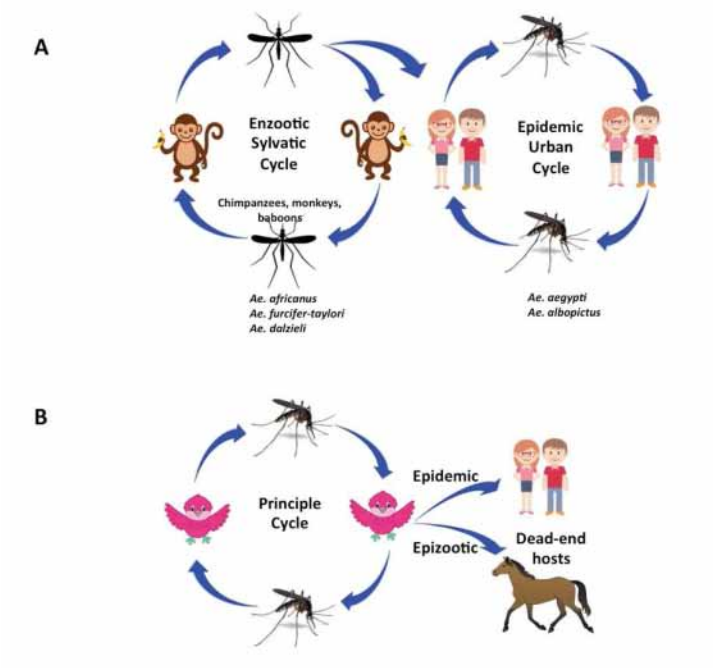

Figure 4. Arbovirus transmission cycles. Adapted from (Anez et al., 2012, Weaver and Barrett, 2004).

Anthropophilic vector, replication-competent human-biting animal, is required for becoming arbovirus endemic leading epidemic. The world distribution of each arbovirus is thus limited to the geographic range of their vectors. However, arboviruses have been proven to be adaptable to new vectors and hosts over time. Hence, the virus able to expand throughout the globe not only due to the vector adaptation but also globalization, climate change or failure of vector control (Anez et al., 2012).

1.6 Type I IFN induction

As a typical adaptive immune response does not develop immediately following infection, the innate immune system seems to be capable of controlling viral infection. Over the past 50 years has defined type I interferons (IFNs), IFN-α and IFN-β, a major component of this innate immune response, as central to the control of viral infection. IFN-α and IFN-β are mainly produced by leukocytes and fibroblasts, respectively (Hayes, 1981). The type I IFN production is triggered through pattern-recognition receptor (PRRs), upon recognition of a viral pathogen-associated molecular pattern (PAMP). The major PRRs encoded by nearly every mammalian cells are the retinoic-acid inducible gene-I (RIG-I)-like receptors (RLR), Toll-like receptors (TLR), and the nucleotide oligomerization domain (Nod)-like receptors (NLR) (Takeuchi and Akira, 2009). Two types of PRRs that recognize viral PAMPS have been identified; TLRs (reside in the plasma membrane or the endosomal compartments) and RLRs (reside in the cytoplasm) (McCartney and Colonna, 2009, Pichlmair and Reis e Sousa, 2007).

Single-stranded RNA genome, and may generate double-stranded RNA, can activate IFN pathway (Figure 5). The RNA triggers host PRRs, including TLR3, TLR7 and TLR8, and cytoplasmic RLR, RIG-I and MDA5, which activate downstream adaptor molecules (TRIF, MyD88, and CARDIF) to induce nuclear translocation of IRF3/7 leads to the activation of type I IFN and the transcription of cytokines and chemokines (White et al., 2011). The inflamasome may also induce interleukin (IL)-1β production by infected cells. MyD88 also acts as an adaptor for IL-1β receptor, which could be activated by the secretion of IL-1β from infected cells, thereby inducing type I IFN in non-infected cells (Schilte et al., 2010). IFN-α and IFN-β are secreted from infected-cell and bind to the IFN-α/β receptor (IFNAR) complex in an autocrine or paracrine manner. Consequently, triggering of the canonical pathway for IFN type I signalling through tyrosine kinase 2 (Tyk2) and Janus kinase 1 (Jak1), activates signal transducer and activator of transcription 1 (JAK-STAT) activation results in an assembly of the IFN-stimulated gene factor 3 (ISGF3) complex. The ISGF3, composed of STAT1-STAT2 dimers and IRF9, then translocates to the nucleus and binds specific DNA sequences known as interferon stimulated response elements (ISRE) to initiate production of antiviral ISGs (Horvath, 2004).

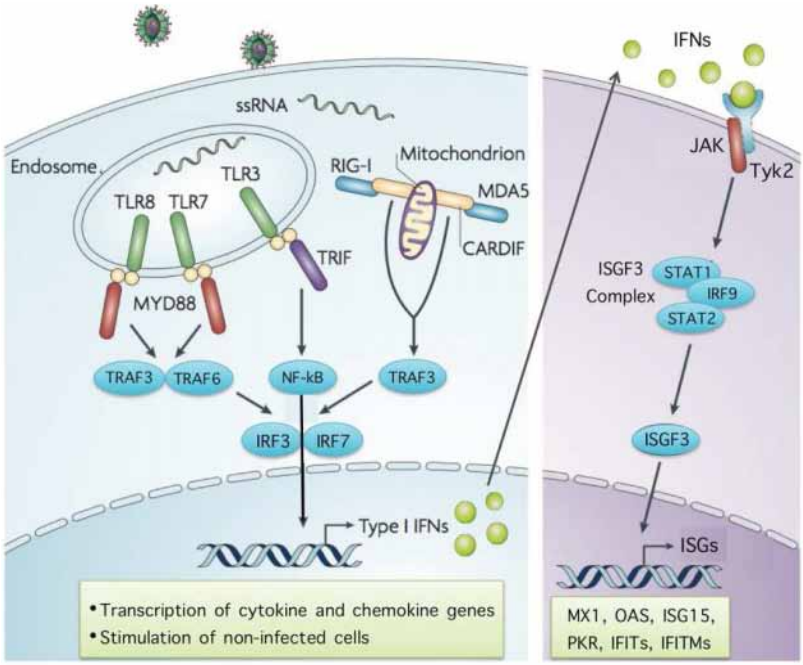

Figure 5. Innate immune signaling via type I IFNs. Adapted from (Quicke and Suthar, 2013, Schwartz and Albert, 2010)

Hundreds of interferon stimulated genes (ISGs) are transcribed, and their products set up an antiviral state in the IFN-treated cell (Sadler and Williams, 2008). Representative and well-studied ISG members with specific or broad antiviral activities include RIG-I, MDA5, MX2, IRF1, IRF3, IRF7, IRF9, IFITM3, ISG15, and OASL (Der et al., 1998, Schneider et al., 2014, Schoggins, 2014). Four ISGs: IFITM2, IFITM3, viperin, and ISG20, are antiviral against DENV, and the IFITM proteins is proposed to target binding, entry, or nucleocapsid uncoating both WNV and DENV infections. However, the protein kinase R (PKR) can inhibit WNV infection but not DENV infection (Brass et al., 2009, Jiang et al., 2010).

Previously, it has been shown that OAS2, ISG15 and MX1 are activated in CHIKV and ZIKV human skin-infected cells (Ekchariyawat et al., 2015, Hamel et al., 2015). These ISGs require further study to confirm their inhibitory activity in *vivo* and determine their mechanisms of action. In addition, PKR and OAS3 are up-regulated during CHIKV replication leading to translation inhibition, apoptosis, and degradation of ssRNA (Bréhin et al., 2009, Clavarino et al., 2012, White et al., 2011). IFIT1 acts as both an antiviral effector molecule and inducer of innate immunity against alphaviruses including CHIKV (Reynaud et al., 2015). Moreover, IFITM3 inhibits CHIKV and ZIKV infection and restrict pathogenesis in the latter virus (Poddar et al., 2016, Savidis et al., 2016).

1.7 Clinical manifestations and chronic sequelae

The global distribution of diseases transmitted by arboviruses represents a challenge for clinicians due to the similarities between their manifestations and sometimes overlapping symptoms especially the symptom of DENV, CHIKV and ZIKV infection (Table 2). In dengue, the disease has three phases: febrile, critical, and convalescence or recovery with current three classifications: dengue without warning signs/probable dengue, dengue with warning signs and severe dengue (WHO, 2009).

Table 2. Clinical comparison of Dengue, Chikungunya and Zika.
Adapted from (Furuya-Kanamori et al., 2016, Ioos et al., 2014, Patterson et al., 2016)

Disease	Dengue	Chikungunya	Zika
Fatality rate	0.5-3.5%	0.1%	ND*
Fever	++++	+++	+++
Myalgia/arthralgia	+++	++++	++
Edema of extremities	0	0	++
Maculopapular rash	++	++	+++
Retro-orbital pain	++	+	++
Conjunctivitis	0	+	+++
Lymphadenopathies	++	++	+
Hepatomegaly	0	+++	0
Leukopenia/thrombopenia	+++	+++	0
Hemorrhage	+	0	0

* ND, No Data

It is known that, infection with CHIKV, around 20% adults, 40% neonates and infants are asymptomatic (Ritz et al., 2015). Atypical presentations and complications of the disease predominate in patients with cardiovascular comorbidities, neurological comorbidities, respiratory comorbidities, systemic arterial hypertension and prior administration of non-steroidal anti-inflammatory drugs (Economopoulou et al., 2009). In case of ZIKV, approximately 20% of individuals who become infected progress to a clinically apparent febrile illness, although hospitalization is rare (Duffy et al., 2009). There are no reported fatal cases of ZIKV in otherwise healthy people. However, ZIKV-associated mortality has been described in patients with complication, such as sickle cell disease, congenital ZIKV infection and post-ZIKV Guillain- Barré syndrome which can be fatal (Arzuza-Ortega et al., 2016).

1.8 Laboratory Diagnosis and Detection

The co-circulation of multiple *Aedes*-transmitted diseases with similar epidemiology, which often result in clinically indistinguishable febrile syndromes, underscores a desperate need for point-of-care diagnostic tests that can differentiate between them. Antibody detection tests can distinguish between the alphaviruses (CHIKV, VEE, MAYV, and RRV) and the flaviviruses (DENV, ZIKV, YFV, WNV, and JEVs). However, serological tests such as IgM ELISA and even neutralisation assays are not reliable for identifying the cross-infection flaviviruses. The most reliable tests for virus detection are nucleic acid tests such as reverse transcription-PCR and non-structural glycoprotein-1 (NS1) ELISA, which detect acute-phase infections, although NS1 ELISA is not available for all of these viruses. There is a crucial need, therefore, to develop sensitive and specific multiplex diagnostic tests that are reliable for differential diagnosis of these viruses (Wilder-Smith et al., 2017).

1.9 Dengue, Chikungunya and Zika virus co-infection in humans

Since DENV, ZIKV and CHIKV using the same *Aedes* mosquitoes as vectors, exploit sylvatic and urban cycles as mode of transmission and as the endemic areas of these viruses often overlap, co-circulation of the virus has been reported in various geographic areas, including Southeast Asia and intertropical Africa (Figure 6).

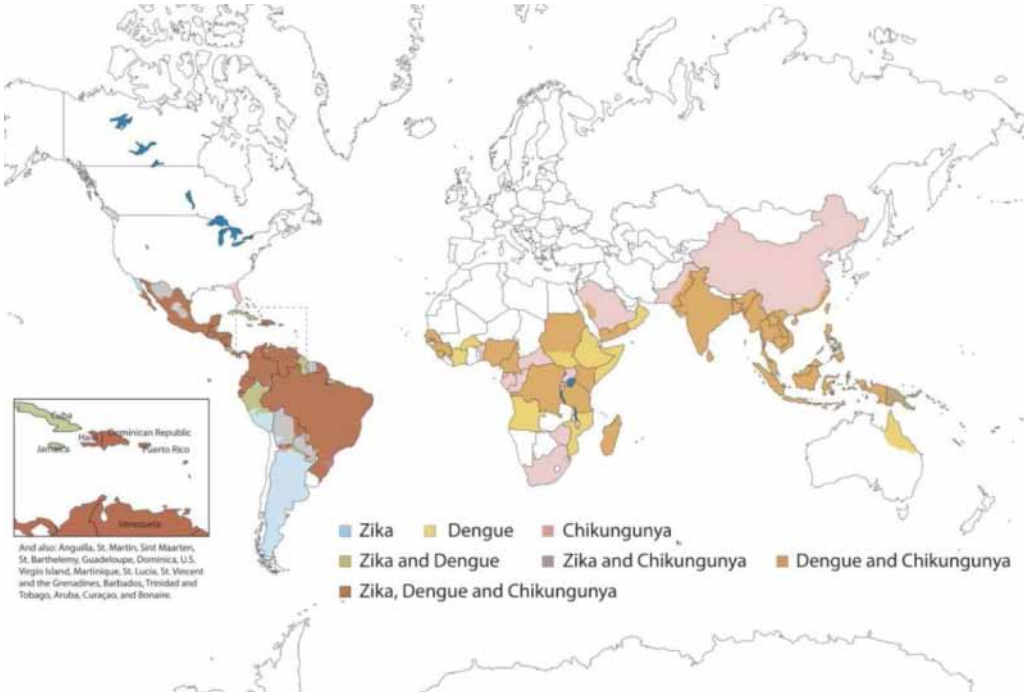

Figure 6. Global distribution of DENV, ZIKV and CHIKV. (Patterson et al., 2016)

Evidence for CHIKV-DENV co-infection has been found in Angola, Gabon, India, Madagascar, Malaysia, Myanmar, Nigeria, Saint Martin, Singapore, Sri Lanka, Tanzania, Thailand and Yemen; these constitute only 13 out of the 98 countries/territories where both viruses have been reported (Furuya-Kanamori et al., 2016). To date, the number of reported cases of CHIKV-DENV co-infection is increased considerably during the past 10 years (Perera-Lecoin et al., 2016). The most recent cases were diagnosed in South America, India and Nigeria in 2013-2014. Of note, two of these cases corresponded to infected travelers returning to Portugal and Germany after being infected in Angola and India, respectively (Parreira et al., 2014, Schilling et al., 2009), raising concern about the possible spread of co-infection cases in Europe where *Ae. albopictus* is present.

The co-infection of CHIKV-ZIKV was first reported in a patient, with a high serum titer of CHIKV, presented with a prolonged "CHIKV-like" illness characterized by urinary inflammation (Baishya et al., 2010) and prominent arthralgia (Weaver and Lecuit, 2015) that persisted for weeks, resulting in repeated emergency room visits. Whereas, the second patient shown a higher titer of ZIKV, with a classic "ZIKV-like" presentation consisting of fever, rash, myalgia, and conjunctivitis (Fauci and Morens, 2016). After that, Sardi and colleges reported a mosquito-borne CHIKV-ZIKV co-infections in 13.3% acutely symptomatic individuals with established ZIKV infection in Bahia state, Brazil using metagenomic next-generation sequencing (Sardi et al., 2016). Recently, a 40 years old woman who return to the Americas, a traveller to Colombia, developed an illness and arthralgia with RT-PCR positive only for ZIKV. Interestingly, cytopathic effects of both CHIKV and ZIKV were identified, concomittantly, phylogenetic analysis of the viruses shown the related to strains found in Colombia (Cherabuddi et al., 2016).

The DENV-ZIKV-CHIKV co-circulating is also found in many parts of world including the Americas (Patterson et al., 2016). Recently, in 2015, Villamil-Gomez and coworkers reported co-infection with the three viruses in a 49 year old male patient from Columbia. The patient was reported with febrile illness with 38^{o}C temperature, conjunctivitis, maculopopular rashes etc. The blood sample collected from the patient was positive for DENV and CHIKV IgM antibodies. While, the sample was positive by RT-PCR for ZIKV and DENV. Fortunately, no synergistic effect of these infections were observed in the patient. (Villamil-Gómez et al., 2016, Villamil-Gómez and Rodriguez-Morales, 2017). Additionally, ZIKV, CHIKV and DENV RNA were detected from cerebrospinal fluid of patients admitted to the Hospital Luis Vernaza (Guayaquil, Ecuador), notably, the breadth and significance of neurological manifestations associated with these infections (Acevedo et al., 2017).

The acute illness caused by these viruses, characterized by fever, rash, myalgia, arthralgia, and conjunctivitis, is nonspecific, and differential diagnosis on the basis of clinical findings alone is challenging. Later infectious sequelae include chronic arthritis for CHIKV (Weaver and Lecuit, 2015) and encephalitis, immune-mediated syndromes, and stroke for DENV (Carod-Artal et al., 2013). Recently, the association between ZIKV infection and severe fetal complications such as microcephaly in pregnant women has been established (Rasmussen et al., 2016), and the virus has also been linked to neurological complications such as Guillain-Barré syndrome (Cao-Lormeau et al., 2016).

Regarding transmission, *Ae. aegypti* has historically been understood to be the vector of greatest public health significance for both CHIKV and DENV. We found no evidence for a substantial role of any alternative vector species prior to 2004. Although *Ae. aegypti* constituted the main vector species in Kenya during the major 2004 outbreak (Kariuki Njenga et al., 2008, Sang et al., 2008), *Ae. albopictus* was the principal vector in succeeding epidemics in Gabon (Caron et al., 2012, Leroy et al., 2009), Madagascar (Ratsitorahina et al., 2008) and La Réunion (Delatte et al., 2008). Initially study has been reported that an artificial infection experiment of the same *Ae. albopictus* mosquito could simultaneously be infected with CHIKV and DENV (Vazeille et al., 2010). The finding concomittant with a naturally co-infected of the same mosquito which was discovered during outbreak of both viruses in Gabon, in 2010 (Caron et al., 2012). Thus, the different replicative strategies, assembly compartments and release mechanisms used by flaviviruses and alphaviruses, may allow these viruses to replicate simultaneously without a substantial overlap in their cellular requirements.

1.10 Disease prevention and control

The combination of mosquito interventions with vaccination becomes interesting on the basis of the growing consensus that no single intervention will be sufficient to reduce disease from the increasing number of arboviruses (Reiner et al., 2016). Vaccination can increase herd immunity, making it easier to sustain reduced virus transmission with vector control. Vector control can complement a vaccine by lowering the risk of infection, making vaccine delivery goals easier to achieve.

Vector control: Initially, application of insecticide trials with indoor residual spraying and space spraying were associated with reduced or eliminated *Aedes spp.* populations and effectively decreased epidemic yellow fever and dengue incidence. Unfortunately, these successes were unsustainable exceptions.

Epidemic dengue virus returned to Latin America and after 15-20 years of control. The development of technologies that can be applied during the day to protect against mosquito bites should similarly be a priority (Orsborne et al., 2016). Various new approaches in development show promise for enhanced disease prevention including infecting *Aedes* mosquitoes with the bacteria of the genus *Wolbachia* either to decrease their capacity to transmit viruses or to reduce mosquito populations and advances in genetic engineering technology (CRISPR/Cas9) that allow highly efficient, targeted transformation and gene-drive systems in mosquitoes (Gantz et al., 2015).

Vaccine: A safe, effective yellow fever vaccine has been available for more than half a century. In late 2015, after decades of research, the world's first dengue vaccine was licensed: CYD-TDV vaccine (Dengvaxia) (Villar et al., 2015). Due to DENV is a complex disease because secondary infections are thought to enhance severity, the CYD-TDV vaccine does not present a simple solution, but despite substantial imperfections, it has public health use as an additional tool (Wilder-Smith et al., 2016). Research on vaccines against CHIKV has been slow, a vaccine based on virus-like particles was successfully tested in a phase 1 trial (Chang et al., 2014). While, replication-defective viral vectors, DNA, inactivated whole virion, or recombinant virus-like particle vaccines for ZIKV are being fast-tracked, and some phase 1 clinical trials are underway (Smith and Mackenzie, 2016).

2. CHIKUNGUNYA VIRUS

Chikungunya virus (CHIKV) is an alphavirus, family *Togaviridae*, that was first isolated in 1952 from febrile human during an outbreak in Tanzania, Tanganyika territory. The disease was given the name chikungunya, a word from the Kimakonde language that translates as "that which bends up", because of the stooped posture and rigid gait of infected individuals (ROBINSON, 1955).

2.1 Classification

Using complete genome sequences of CHIKV obtained from GenBank, genetic study shows that the virus has evolved into 4 distinct genotypes with distinct antigenic characteristics (Figure 7). Four lineages compose of West African (WA), East/Central/South African (ECSA), Asian and the Indian Ocean lineage (IOL or Asia) and Indian Ocean outbreak emerged from the ECSA lineage in 2004 (Volk et al., 2010, Weaver and Forrester, 2015). WA strain mainly distributes enzootic in Western Africa while ECSA lineage continued to circulate out of Africa, evolving independently of the ECSA lineage into a distinct Asian genotype (Volk et al., 2010). The genotypes likely indicate independent evolution of the virus in historically isolated areas. However, recently, Lanciotti and Valadere reported that CHIKV Asian strain was recently introduced into the Caribbean indicated that the movement of virus genotypes has dramatically increased probably as a direct result of increased movement of humans and increased commercial trade (Lanciotti and Valadere, 2014).

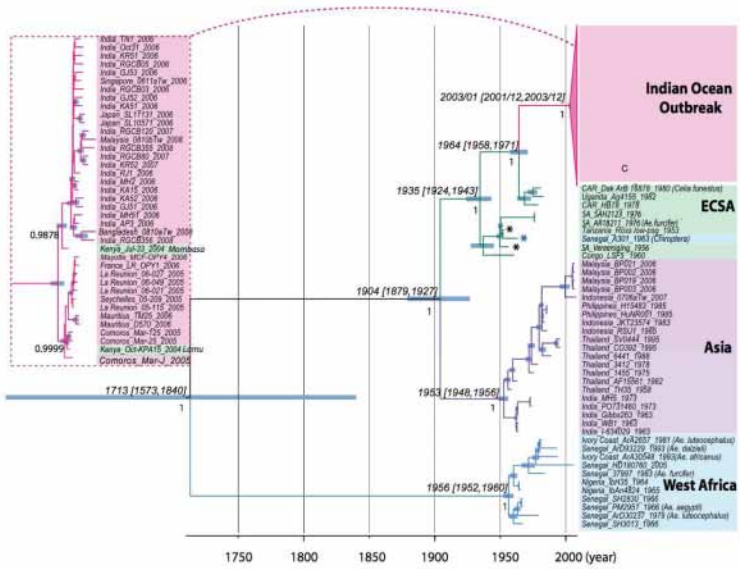

Figure 7. Chikungunya Phylogenetic tree. (Weaver and Forrester, 2015)

2.2 Geographic distributions and Outbreaks

During the last 50 years, CHIKV has distributed and caused a number of outbreaks in East Africa, South Africa and Southeast Asia (Schuffenecker et al., 2006) (Figure 8). The large human outbreaks were described in Indian Ocean islands including Mauritius, Mayotte, Madagascar and La Réunion Island. The most severe outbreak was in 2005-2006 in Réunion Island which 33% of the people infected (Josseran et al., 2006). Concomittantly, in the same period, it was outbreak in India during affected morethan 1.3 million people (Arankalle et al., 2007). This have been attributed to circulating strains from the IOL, a newly emerged subgroup within the ECSA clade (Volk et al., 2010).

Outbreaks then spread to other Southeast Asian countries including Singapore (Ng et al., 2009) and unexpected in European countries, such as Italy in 2007 (Rezza et al., 2007) and France in 2010 and 2014 (Delisle et al., 2015, Grandadam et al., 2011). The outbreaks in Europe caused by an ECSA genotype. Although these outbreaks have been sporadic without extensive spread, the presence of *Ae. albopictus* has raised concerns about the potential for the virus to establish endemicity in Southern Europe (Gould et al., 2010, Rezza et al., 2007). Sporadic outbreaks are still being reported, in December 2013, it was confirmed that CHIKV was being locally transmitted in the Americas for the first time in the French Caribbean dependency of St. Martin (Leparc-Goffart et al., 2014). Since then, over 2 million suspected cases caused by endemic transmission have occurred in almost 50 countries, including 12 cases of autochthonous transmission in the Americas (CDC, 2017a).

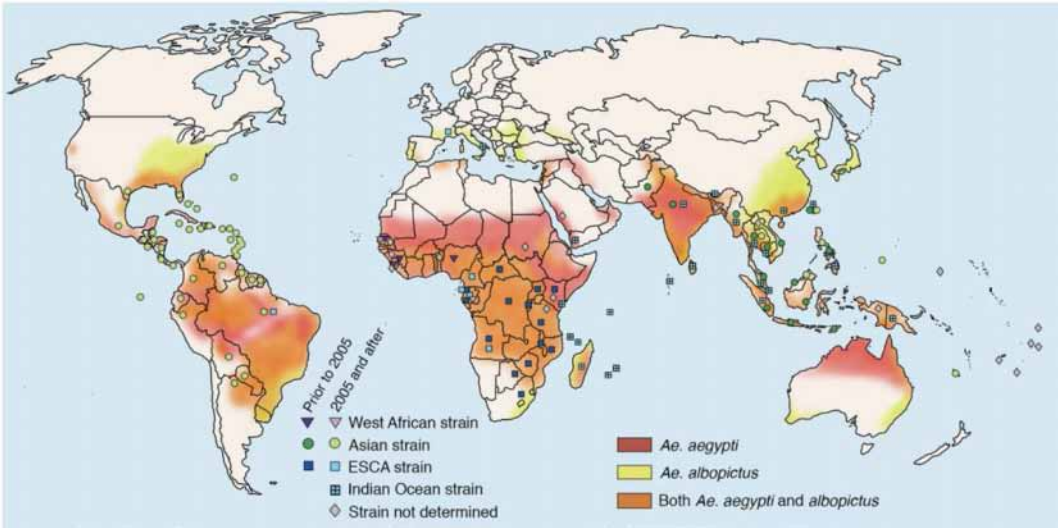

Figure 8. Geographic distribution of CHIKV and its primary vectors. (Silva and Dermody, 2017)

2.3 Viral structure

CHIKV comprises small, spherical, icosahedral nucleocapsid, enveloped viruses with single stranded (ss), positive-sense 49S RNA approximately 11.7 kb in length. The viruses contain two large open-reading frames (ORFs): the 5′ two thirds of the genome (7.6 kb) encodes four nonstructural proteins (nsPs; nsP1-nsP4); the 3′ third (4.1 kb) encodes five structural proteins (sPs; Capsid, E3, E2, 6K, and E1) (Strauss and Strauss, 1994) (Figure 9).

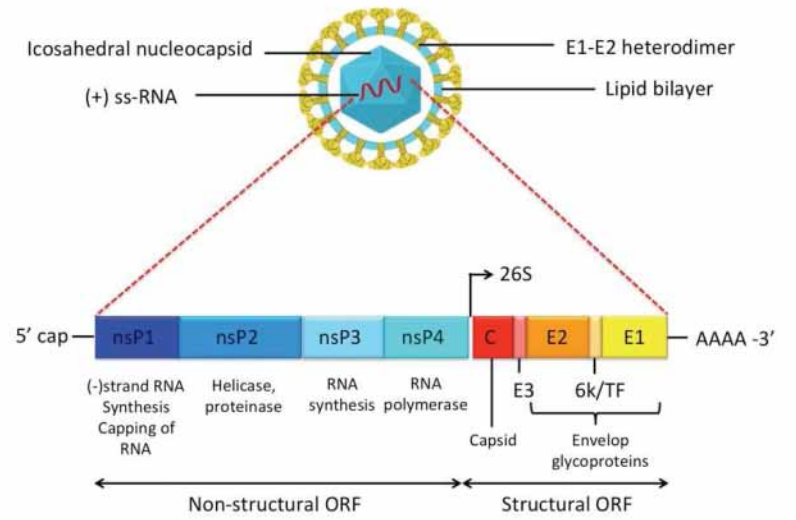

Figure 9. Alphavirus genome structure. Adapted from (Weaver and Lecuit, 2015)

2.3.1 Structural proteins

E1 is responsible for fusion of the viral membrane with the host endosomal membrane, and E2 is involved in receptor binding and the subsequent receptor-mediated endocytosis. While, E3 is required for efficient particle assembly, mediating both spike folding and spike activation for viral entry. Small 6K protein is important in glycoprotein trafficking and virion assembly. The capsid functions as a serine protease with His141, Asp163 and Ser215 forming the catalytic triad (Jose et al., 2009).

2.3.2 Non-structural proteins

Nonstructural protein 1 is the membrane anchor of the replication complex (RC) which is composed of Rossman-like methyltransferase (MTase) and guanylyl transferase (GTase), necessary for capping of the viral RNA. During viral replication, nsP2 has three important functions.

It acts as a helicase to unwind RNA secondary structure formed, a RTPase to enable viral RNA for capping reaction and a protease to process the non-structural polyprotein. The nsP3 protein is essential for RNA synthesis. Fros et al., reported that the interaction between CHIKV nsP3 and host Ras-GAP SH3 domain-binding protein (G3BP) prevents further assembly of stress granules which has antiviral properties (Fros et al., 2012). Whereas, nsP4 is solely accountable for the RNA-synthetic properties of RC (Rupp et al., 2015).

2.4 Viral Replication

2.4.1 Receptor-mediated endocytosis

The first step of infection of CHIKV is bind to host cell receptors which based on the cell types. Receptor binding is facilitated by the E2 domain 2 glycoprotein of CHIKV (Ashbrook et al., 2014, Smith et al., 1995). To date, prohibitin (PHB), phosphatidylserine (PtdSer)-mediated virus entry-enhancing receptors (PVEERs), and glycosaminoglycans (GAGs) have been suggested as CHIKV receptor proteins in mammalian cells (Moller-Tank et al., 2013, Silva et al., 2014, Wintachai et al., 2012) and ATPsynthase β subunit in mosquito cells (Fongsaran et al., 2014). Notably, CHIKV infection can proceed in absence of these proteins, indicating that these proteins facilitate the initial interaction with the cell surface rather than virus uptake (Marsh and Helenius, 2006, Salvador et al., 2009).

2.4.2 Life cycle

The virus binds to a host receptor via the E1 and E2 glycoproteins. Following endocytosis, the acidic environment of the endosome triggers conformational changes in the viral envelope which mediates virus-host cell membrane fusion (Kielian et al., 2010). This allows cytoplasmic delivery of the core and release of the viral genome. Two precursors of nsPs are translated from the viral mRNA, and cleavage of these precursors generates nsP1-nsP4. These proteins assemble to form the viral replication complex, which synthesizes a negative-strand RNA intermediate served as the template for subgenomic (26S) and genomic (49S) RNAs synthesis.

The 26S RNA drives the expression of the C-pE2-6K-E1 polyprotein precursor. The protein precursor is then processed by autoproteolytic serine protease. The capsid is released, and the pE2 and E1 glycoproteins are generated by further processing.

pE2 and E1 associated in the Golgi and are exported to the plasma membrane, where pE2 is cleaved into E2 and E3. Binding of the viral nucleocapsid to the viral genome and the recruitment of the membrane-associated envelope glycoproteins promote viral assembly. The assembled alphavirus particle, with an icosahedral core, buds at the host cell membrane (Figure 10).

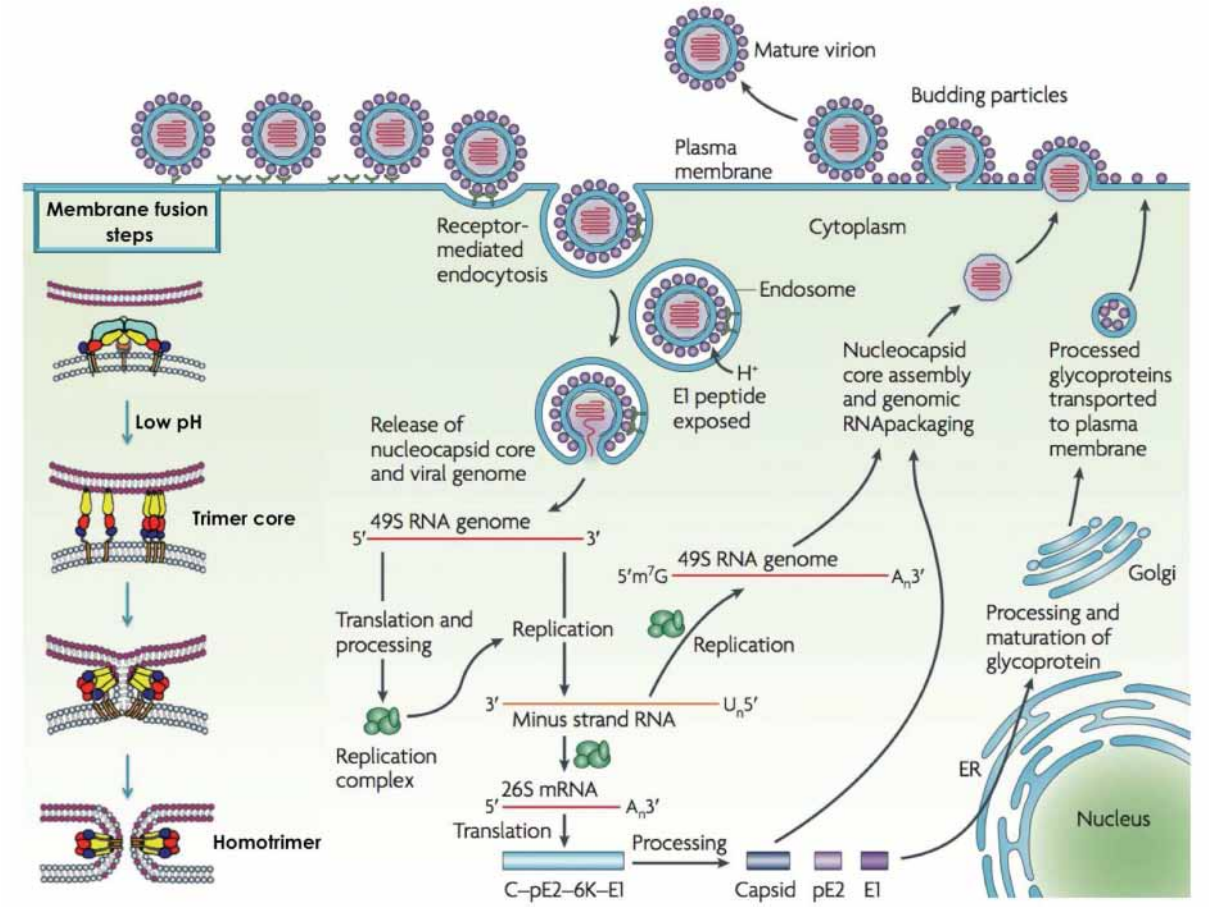

Figure 10. Alphavirus replication cycle. Adapted from (Kielian et al., 2010, Schwartz and Albert, 2010).

2.5 Mechanisms of immune evasion

Sucessful of virus replication in infected hosts relies not only on their replicative machinery but also on their ability to interfere with the antiviral. The outbreaks of CHIKV throughout the world emphasize the requirement of its vaccines or antivirals. Understanding the mechanisms of the virus modulated host cell antivirals or stress responses will advantage for the antiviral research development. CHIKV employed several processes to escape host cells responses as shown in Figure 11.

NsP2 induces the degradation of RNA polymerase II subunit RPB1 in host cells nucleus, which results in general host cell transcriptional shut-off and consequent cytopathic effects in mammalian cells (Akhrymuk et al., 2012). This effect was confirmed by the fact that during CHIKV infection, most subunits of the RNA polymerase II complex were progressively degraded and second point mutations of nsP2, at positions P718 and KR649 or D711, establish noncytopathic of CHIKV RNA replication (Fros et al., 2013, Pohjala et al., 2011, Treffers et al., 2015). In addition, nuclear localization of nsP2 completely reinstated the JAK-STAT signaling pathway resulting in inhibited IFNs production (Fros et al., 2010).

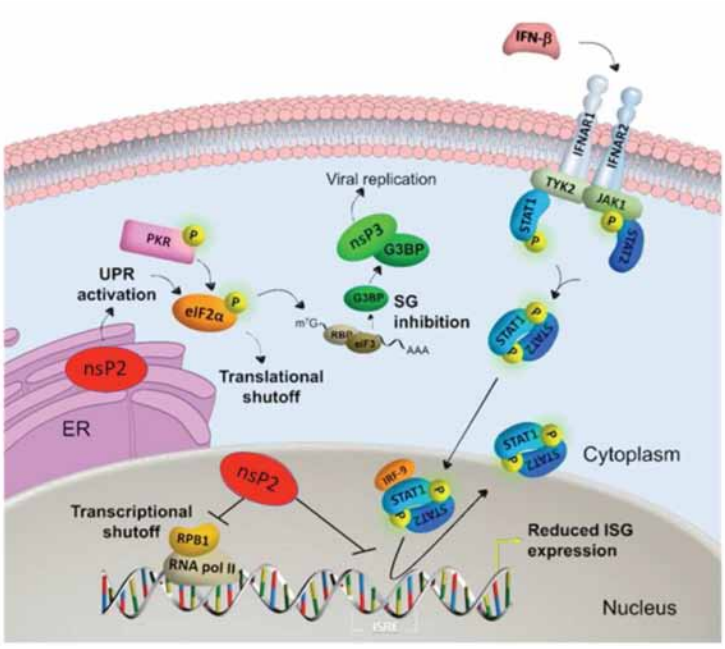

Figure 11. Mechanism of immune invasion by Chikungunya virus. Adapted from (Fros and Pijlman, 2016)

Recently, Smith suggests that the unfolded protein response (UPR) can support important antiviral responses (Smith, 2014). CHIKV nsP2 proteins activate the UPR in the endoplasmic reticulum (ER) and together with kinases such as protein kinase R (PKR) phosphorylate eukaryotic translation initiation factor 2α (eIF2α) (Fros et al., 2015b, White et al., 2011). Phosphorylated eIF2α resulting in host cells translational shut-off allows and promotes the translation of viral proteins. Moreover, the stress granules (SG), antiviral RNA binding proteins, is inhibited by CHIKV nsP3/G3BP/Rin exerts a positive effect on viral replication both in vertebrate cells and the mosquito vector (Fros et al., 2012, Fros et al., 2015a, Scholte et al., 2015).

3. INTRACELLULAR CHOLESTEROL TRAFFICKING

Cholesterol, four-ring core structure lipid, is biosynthesized by all animal cells, via *de novo* synthesis as well as from dietary sources. Cholesterol is important for maintain structural integrity and fluidity of cell membranes (Grundy, 1983). Cholesterol is enriched in the plasma membrane, where it typically accounts for 20-25% of the lipid molecules, with various phospholipids, sphingomyelin and glycolipids making up the remainder. It is also abundant in the endocytic recycling compartment, Golgi complex and endoplasmic reticulum (ER), even in the lesser extent (Lange, 1991).

Viruses have evolved complex and dynamic interactions with their host cell. They share a common feature dependence on host cell factors to complete their replicative cycle. Among the cellular factors required by viruses, lipids play an important role on viral infection especially enveloped viruses (Lorizate and Kräusslich, 2011, Stapleford and Miller, 2010). Furthermore, because cholesterol has profound physical effects on the membranes, it is not surprising that membrane cholesterol also dramatically affects both viral fusion and fission steps.

3.1 Cholesterol and viral entry

To start the replicative cycle, viral entry into a host cell involves the attachment of the virus particle to a specific receptor(s) located on the cell surface, prior to the introduction of the viral genome within the host cell. Plasma membrane contains lipid microdomains termed «lipid rafts», cholesterol-rich region with glycosphinglolipids and glycophosphatidylinositol (GPI) (Jacobson et al., 2007). Many viruses, such as severe acute respiratory syndrome (SARS), herpes simplex virus and JEV use lipid raft to entry into host cells (Bender et al., 2003, Das et al., 2010, Glende et al., 2008). To release viral genome, the fusion between viral particles and host cells membrane are required. The presence of cholesterol on the target membrane promotes WNV membrane fusion activity, while, depletion of cellular cholesterol levels β-cyclodextrin resulted in a 100-fold reduction of the viral production (Medigeshi et al., 2008, Moesker et al., 2010). Similary, both cholesterol and sphingolipids are required for alphavirus fusion (Kielian et al., 2010). CHIKV, DENV and JEV and also need cholesterol for their entry step. As the virus fails to enter their host cells after membrane cholesterol depletion by methyl β-cyclodextrin (Bernard et al., 2010, Lee et al., 2008).

3.2 Cholesterol and viral replication

Virus co-opt host cell factors to develop the most adequate environment for their replication, for example, positive senses RNA viruses, share common characteristics of host cells mRNA, take this advantage to translate into their viral proteins (Stapleford and Miller, 2010). Besides, morphological changes on membrane shape induced by virus increases the local concentration of specific cellular and viral factors necessary for replication. It have been reported that DENV and WNV improve their multiplication by induce membrane remodelling, which hiding their viral components from the host innate immune system. (Ambrose and Mackenzie, 2011, Muñoz-Jordan et al., 2003) (Mackenzie et al., 2007, Miller and Krijnse-Locker, 2008).

Recently, it has been reported that cholesterol is required for the formation of large and stable Phosphatidylinositol 4-Phosphate (PI4P)-enriched lipid domains which planty in replication organelles, ER, Golgi and trans-Golgi network (TGN) (Figure 12) (Jiang et al., 2014). PI4P/cholesterol-enriched membranes facilitate viral RNA synthesis, take place in modified membrane structure, by helping dock and concentrate viral replication proteins, by stimulating viral enzymatic reactions and by generating high curvature membrane pockets that can concentrate and segregate viral replication machinery away from the host innate immune defenses.

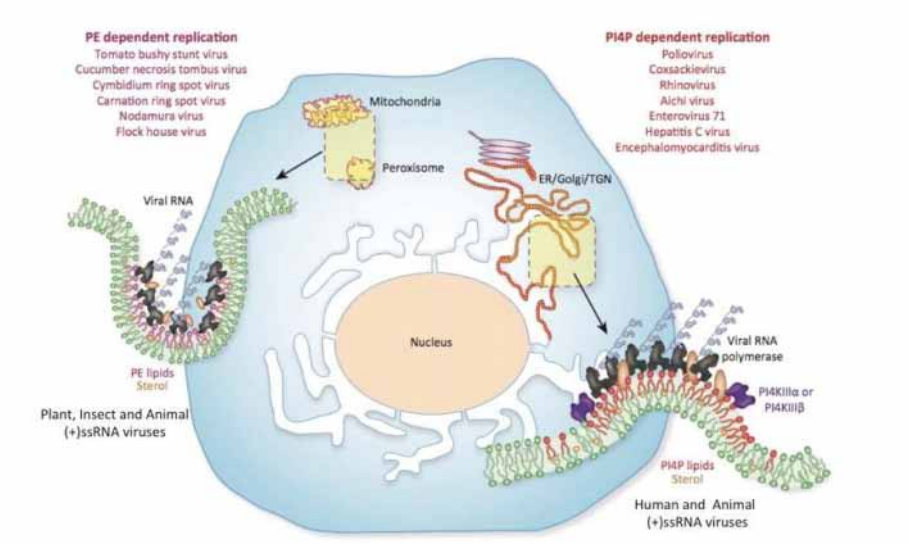

Figure 12. Cholesterol and replication organelles. Adapted from (Altan-Bonnet, 2017)

3.3 Cholesterol and viral morphogenesis

After replication and assembly into new viral particles, the viruses can co-opt membrane as carrier to be transported out and be transmitted to other suseptible hosts. Cholesterol and lipid raft microdomains play an important role on the assembly of a variety of viruses. Human immunodeficiency virus (HIV) and influenza virus exploit lipid rafts for assembly and budding (Freed, 2015, Scheiffele et al., 1999). Normally, Influenza virus buds from membrane lipid rafts. The lipidome of this virus has been analyzed for viruses budding from the apical membrane of polarized cells. The study revealed that the apical cellular membrane was enriched in sphingolipids and cholesterol, whereas glycerophospholipids were reduced, and storage lipids were depleted compared with the whole-cell membranes. These results are consistent with an accumulation of lipid rafts at the membranes where the virus buds. In addition, the virus membrane exhibited a further enrichment of sphingolipids and cholesterol when compared with the donor membrane at the expense of phosphatidylcholines (Gerl et al., 2012). Recently, Bajimaya and colleges reported that cellular cholesterol is required for assembly and formation of type 1 parainfluenza virus and suggest that cholesterol could be an attractive target for antiviral agents against this virus (Bajimaya et al., 2017).

Lipid droplets are ER-related lipid rich cellular organelle that regulate the storage and hydrolysis of cholesterol and acyl-glycerol to prevent lipotoxicity. The organelle is also involved in protein quality management, pathogenesis, immune responses, and, potentially, in neurodegeneration (Wang, 2016). It has been hypothesized that lipid droplets associate with DENV C protein to form «lipoviroparticles», which would constitute a novel step on the viral life cycle. Accordingly, the dissociation of the C protein from lipid droplets inhibits viral production, but not RNA replication, supporting the role of these organelles on the morphogenesis of DENV (Carvalho et al., 2012, Samsa et al., 2009).

3.4 Targeting cholesterol as an antiviral strategy

The dependence on cholesterol for different processes during viral infection provides a suitable target for antiviral strategies. In this regard, statins (3-HMG-CoA reductase inhibitor), commonly used in treatment of cardiovascular disease, highlight the feasibility of lipid-based therapeutics (Gilbert et al., 2005, Gower and Graham, 2001). Beneficial effects derived from treatment with different statins on HCV and diverse influenza strains infection have also been reported in both animal models and human studies (Bader et al., 2008, Brett et al., 2011, Liu et al., 2009, Mihăilă et al., 2009).

Lovastatin, also blocks prenylation pathways by directly inhibiting 3-HMG-CoA reductase, inhibits WNV replication in Vero cells and RSV infection both in *vitro* and in *vivo* (Gower and Graham, 2001, Mackenzie et al., 2007). Challenge with the same compound affects DENV assembly in cell culture and increases survival rate in infected mice (Martinez-Gutierrez et al., 2014). The clinical success of these inhibitors for human disorders also indicates that inhibitors of lipid metabolism can be safe and effective for human therapy.

4. SAMHD1 PROTEIN

SAMHD1 (Sterile Alpha Motif and Histidine-aspartic Domain containing protein 1) is a protein involved in autoimmune disorder Aicardi-Goutières syndrome (AGS), a genetic encephalopathy with symptoms mimicking congenital viral infection (Rice et al., 2009). Using confocal microscopy, SAMHD1 is predominantly in nucleus, however, a fraction of SAMHD1 is also found in the cytoplasm of primary macrophages and CD4+ T-lymphocytes (Baldauf et al., 2012). It is expressed in all tissues but its restriction activity is confined to differentiated noncycling cells (Laguette et al., 2011).

This protein has been identified as a virus restriction factor that inhibit retroviral replication at distinct stages of the viral life cycle (Laguette et al., 2011). Since then, SAMHD1 becomes the most interested host intrinsic restriction agent not only retrovirus but also hepatits B virus through its dNTPase or RNase activity (Chen et al., 2014, Choi et al., 2015, Ryoo et al., 2014, Sommer et al., 2016). Despite these findings, no information is available regarding the role of SAMHD1 during arbovirus infection.

4.1 SAMHD1 structure and function

SAMHD1 is a 626 amino acid protein composed of two domains, SAM and HD domain, nuclear localization signal (NLS) at N-terminal and a site capable for Vpx (HIV-2/SIVsm accessory protein) interaction at C-terminal, as indicated in (Figure 13). There are four critical residues in NLS (11KRPR14), four residues in the enzymatic HD domain (H167, H206, D207, and D311), and phosphorylation of threonine at residue 529 (T592) (Wu, 2013). SAM domain involves in protein-protein interactions and contributes to SAMHD1 nucleic acid binding while HD domain contains the enzymatic sites crucial for its triphosphohydrolase activity, RNA binding and nuclease activity (Beloglazova et al., 2013).

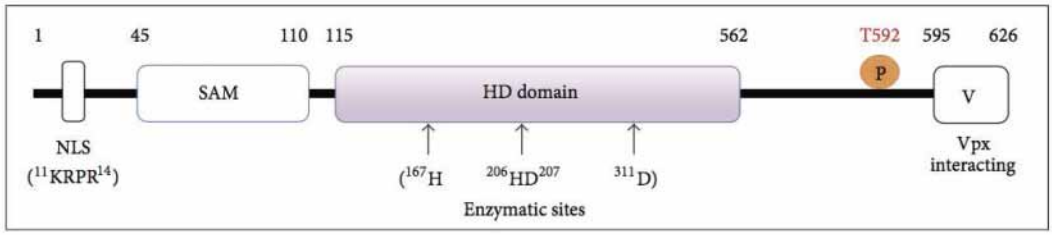

Figure 13. SAMHD1 structure and its function domain. (Wu, 2013)

Two catalytic activities of SAMHD1 via HD domain are proposed (Figure 14A), dNTPase and RNase activities, controlled by D137N and Q548A mutation, respectively (Ryoo et al., 2014). Although SAM domain is not contribute to enzymatic function, it is necessary to the increasing of both dNTP triphosphatase and nuclease activities (Goncalves et al., 2012). Based on the crytal structure analysis, it has been reported that the catalytically active tetramers is required for SAMHD1 dNTPase activity (Ji et al., 2013).

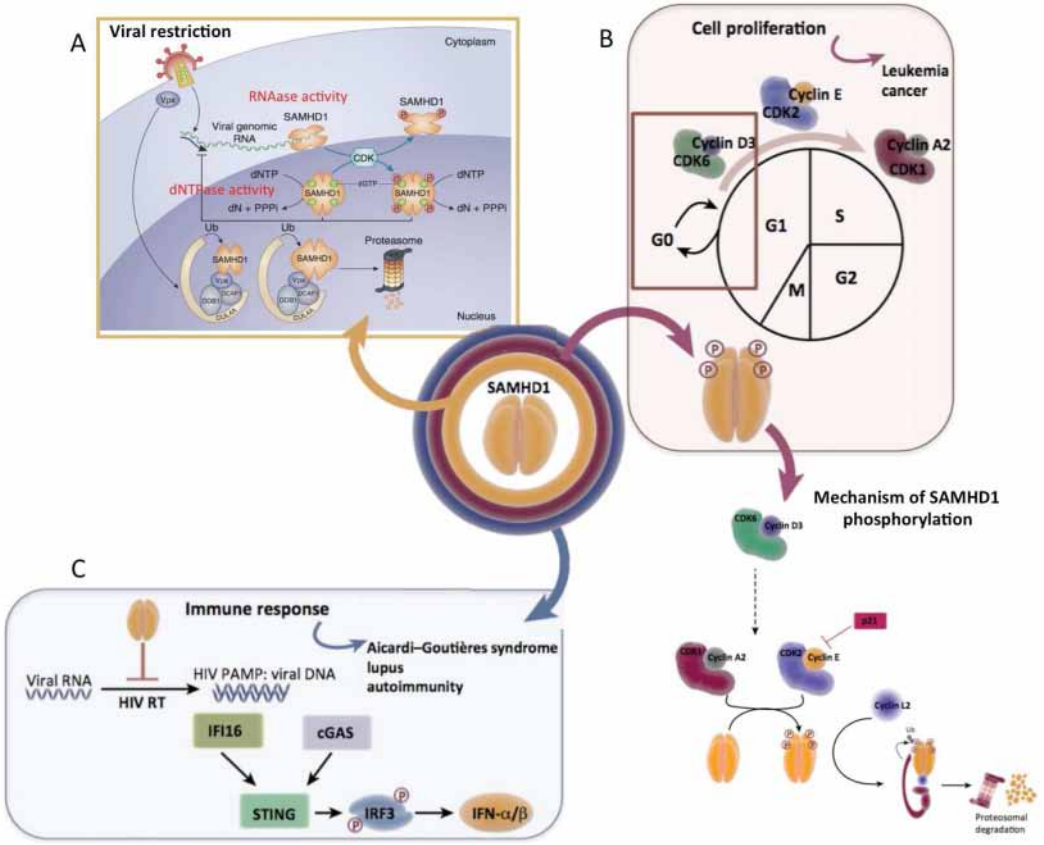

Figure 14. Essential Role involved by SAMHD1. Adapted from (Ballana and Esté, 2015, Simon et al., 2015)

The dNTPase depletes intracellular dNTP pool limited the availability of dNTPs for viral reverse transcription which is needed to generate dsDNA (Goldstone et al., 2011). However, it has been found that phosphorylation of SAMHD1, as well as T592A mutation mimicking phosphorylation, has been shown to abrogate HIV-1 replication without affecting cellular dNTP levels, based on that, dNTP depletion may be either not sufficient for antiviral activity or not required (Cribier et al., 2013, White et al., 2013b). Thus, an alternative mode of action to explain SAMHD1-mediated viral restriction has been proposed, in which, SAMHD1 specifically targets HIV-1 RNA for degradation before it is reverse-transcribed in the cytoplasm (Ryoo et al., 2014).

4.2 SAMHD1 Regulation

SAMHD1 activity can be regulated by a number of factors including degradation by Vpx, phosphorylation state and blocking by type I IFN. As mention in the previous section, SAMHD1 contains a domain at the C-terminus capable of interacting with the HIV-2/SIVsm Vpx accessory protein (Ahn et al., 2012) (Figure 13). Vpx disrupts SAMHD1 function in non-dividing cells and promotes its proteasomal degradation by interacts with the cullin-4-based E3 ubiquitin ligase CRL4/DCAF1 and tethers SAMHD1 into Cull4/CRL4/ DCAF1 complex, leading to ubiquitination proteosomal degradation via the C-terminus of SAMHD1 in the nucleus (Brandariz-Nuñez et al., 2012, Goujon et al., 2008, Hofmann et al., 2012, Laguette et al., 2012). Besides, Vpx can inhibit SAMHD1 activity prior to its degradation (DeLucia et al., 2013).

Although, SAMHD1 is ubiquitously expressed in immune cells, and its expression levels are similar in monocytes and quiescent lymphocytes, differentiated macrophages and activated lymphocytes are still susceptible to HIV-1 infection (Cribier et al., 2013, White et al., 2013b). These characteristics indicate that a post-transcriptional mechanism controls SAMHD1 function in proliferating cells (Figure 14B). Mammalian cell-cycle progression throughout the G1 phase is controlled by signaling pathways regulated by G1 Cyclin-dependent kinase (CDK) 6-cyclinD3 and downstream of CDK1-cyclinA and CDK2-cyclinE couples. Phosphorylation of SAMHD1 by the CDK1 together with CDK2 is associated with deactivation of the restriction activity on HIV-1 replication in primary myeloid and lymphoid cells (Cribier et al., 2013, White et al., 2013b, Yan et al., 2015).

However, the phosphorylation at T592 loss SAMHD1 restriction ablity but not its dNTPase capability (Welbourn et al., 2013). The natural CDK2 inhibitor, CDKN1A (p21), allows SAMHD1 dephosphorylation (Pauls et al., 2014). Cyclin L2 has been reported to target SAMHD1 for proteosomal degradation through the addition of ubiquitin, similarly to HIV-2 Vpx (Yan et al., 2015).

Another factor that control SAMHD1 activity is treatment by anti IFN antibody. Initially a report indicated that, treatment of monocytes by IFN-α or IFN-γ to macrophage-like THP-1 cells prevented Vpx from degrading SAMHD1 (Dragin et al., 2013). Supportively, treating plasmacytoid and myeloid DC, normally high resistant to HIV-1, with an anti-type I IFN antibody cocktails restored Vpx activity to induce SAMHD1 degradation resulting in susceptibility to HIV-1 infection (Bloch et al., 2014). However, the mechanism by which type-I IFN blocked the Vpx-induced degradation of SAMHD1 is still unclear.

4.3 Role of SAMHD1 in the innate immunity

Aicardi-Goutières syndrome (AGS), a rare autoimmune disorder, is characterized by high expression type I IFN in the central nervous system and upregulation of IFN-stimulated genes, reminiscent of chronic viral infection (Lebon et al., 1988, Rigby et al., 2008). The disease can be caused by defects in the genes encoding SAMHD1, TREX1, RNase H2A, RNase H2B, RNase H2C, ADAR or IFIH1 (Crow et al., 2015), all of which are enzymes involved in nucleotide or nucleic acid metabolism. Mutations of SAMHD1 found in AGS patients are located in the enzymatically important HD domain, thus in AGS cells, SAMHD1 are highly permissive to HIV-1 replication when compared to healthy cells (Baldauf et al., 2012, Berger et al., 2011).

Yan et al. reported that TREX1 binds and degrades excess cytosolic HIV-1 DNA that could activate type I IFN expression which, consequently, triggers innate immune responses against viral infection. Thus, HIV-1 may evade innate immunity through the viral DNA degradation (Yan et al., 2010). Similarly, SAMHD1 could control endogenous elements by degrading them or preventing their synthesis, further blunting the innate immune response (Simon et al., 2015). Consistent with this possibility, in SAMHD1-null mice, some ISGs are constitutively induced (Behrendt et al., 2013, Rehwinkel et al., 2013). A model in Figure 14C would explain how SAMHD1 restricts HIV-1 via type I IFN immune response in normal cells which failed to control in AGS patient.

5. MOSQUITO SALIVA

The mosquitoes *Aedes (Ae.) aegypti* [Stegomyia aegypti] and *Ae. Albopictus*, [Stegomyia albopicta] are vectors of several globally important arboviruses, including DENV, CHIKV and ZIKV (Weaver and Reisen, 2010). *Aedes* spp. distributions are now the widest ever recorded; extensive in all continents, including North America and Europe, with more than 3 billion people living in *aedes*-infested regions (Figure 15) (Kraemer et al., 2015).

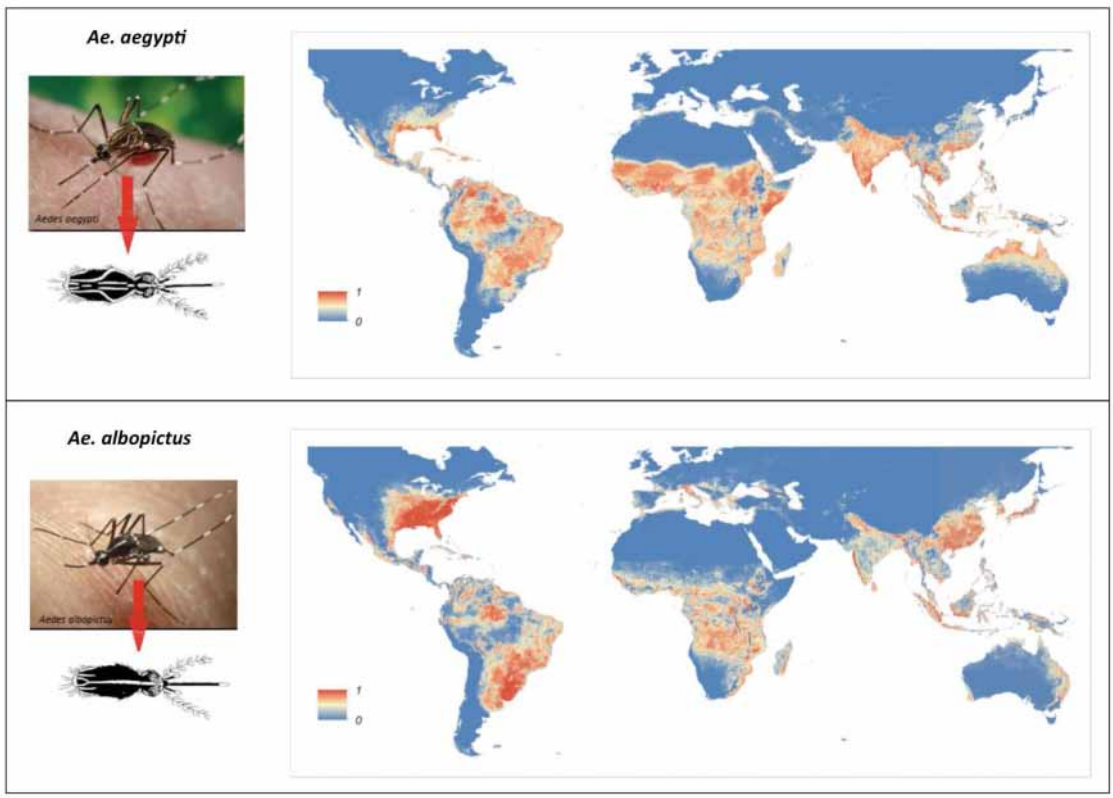

Figure 15. Global distribution of *Ae. aegypti* and *Ae. albopictus* and its morphology.
Adapted from (Kraemer et al., 2015).

Mosquito saliva and their salivary glands are center of the interactions among the pathogen, the vector, and the mammalian host during the arbovirus infection. The pathogen is delivered into the skin of the mammalian host along with saliva, which contains an array of biologically active molecules (Patramool et al., 2012). Mosquito saliva, like that of all hematophagous arthropods, is a pharmacologic cocktail of secreted molecules, especially proteins. These proteins can affect vascular constriction, blood coagulation, platelet aggregation, inflammation, immunity and angiogenesis in order to help the mosquito to successfully obtain a blood meal from a vertebrate host.

The mosquito secretes anti-hemostatic, angiogenic, anti-inflammatory and vasodilatory molecules into their saliva to maintain blood flow during feeding and could also contribute to an optimal transmission of the pathogen by modulating the host immune response (Schneider and Higgs, 2008, Wichit et al., 2016). Besides, Pingen and colleagues reported that inflammation at mosquito bite sites caused an influx of inflammatory neutrophils and pave the way for the chemokine receptor CCR2-dependent entry of myeloid cells that are permissive to viral infection, resulting in more severe infection (Pingen et al., 2016).

5.1 Saliva and host haemostasis

Host cell hemostasis prevents blood loss from a damaged vessel which is activated within a matter of seconds. Damage to blood vessel endothelium first results in vasoconstriction that decreases blood flow at the bite site to limit the hemorrhage (Fontaine et al., 2011). Hematophagous arthropods utilise salivary components which block host vasoconstrictor agents and harbor strong vasodilators in their saliva, such as sialokinin (Champagne and Ribeiro, 1994) and D7 proteins (Calvo et al., 2006) in *Ae.aegypti*.

Suddenly, vascular injury provoked by penetration of the arthropods mouthparts into the host skin is concomitant with the activation of platelets, which aggregate within seconds to form a hemostatic plug using fibrinogen as a connecting agent. In order to inhibit, or to scavenge, platelet-aggregating factors, similar molecules have evolved in different arthropods (Fontaine et al., 2011).

Among these, *Ae. aegypti* secretes an apyrase that inhibits ADP-dependent platelet aggregation (Champagne et al., 1995), D7 and aegyptin proteins can also bind to collagen to inhibit platelet aggregation during mosquito feeding (Calvo et al., 2006, Calvo et al., 2007). This platelet activation paves the way to secondary haemostasis by exposing the surface of activated platelets to coagulation proteins. A cascade of reactions, involving several blood coagulation factors, then leads to the formation of thrombin and blood clotting that abrogates the hemorrhage (Fontaine et al., 2011). The anticoagulant, inhibitor of the 48kDa factor Xa that is found in the saliva of *Ae. aegypti* has been shown to be a member of the serpin family of serine protease inhibitors (Stark and James, 1998).

5.2 Saliva in the modulation of the host immune system

During mosquito blood feeding, the mouthparts first pierce skin and viral particles are released along with saliva, which encounters different types of resident cells. Keratinocytes express PRR including TLR-3 which is known to be responsible for innate immune activation against CHIKV (Briant et al., 2014, Priya et al., 2014) and its loss led to provoked CHIKV pathology (Her et al., 2015). Therefore, mosquito bite with CHIKV inoculation downregulates of TLR-3, as compared to CHIKV alone, points towards the host immune suppression (Agarwal et al., 2016, Cox et al., 2012). This reduction in host immune response could benefit the establishment of CHIKV within vertebrate host. Human dermis is rich in elastin, collagen fibers and extracellular matrix produced by fibroblasts (Briant et al., 2014). Proteolysis of extracellular matrix by serine protease presented in *Ae. aegypti* saliva enhances viral attachment to the cell receptors and facilitates virus infected cell migration (Conway et al., 2014).

Activated APCs (antigen presenting cells) migrate to lymph nodes and present antigen to naive helper T lymphocytes (Th_0 cells) to activate and differentiate into effector Th_1 and Th_2 cells (Fontaine et al., 2011). Th_1 cells secrete inflammatory cytokines like IL-2, IFN-γ, TNF-α and Th_2 cells secrete anti-inflammatory cytokines such as IL-4 and IL-10 which are inhibit viral replication. During mosquito feeding, the D7 proteins inhibit inflammation by sequestering biogenic amines (Calvo et al., 2006). In addition, it has been reported that *Ae. aegypti* saliva reduces murine lymphocyte proliferation, thus reducing TNF-α, IL-2 and IFN-γ secretion by mast cells, led to reduced inflammation in skin that probably enhanced viral migration (Agarwal et al., 2016, Bissonnette et al., 1993).

Another mechanism of immune suppression by mosquito saliva is production of anti-inflammatory cytokine. Up-regulation of IL-4 and IL-10 by CHIKV infection via mosquito bite point towards immune suppression (Agarwal et al., 2016). The immune suppression, induced by mosquito saliva has also been reported in several studies (Cox et al., 2012, Wasserman et al., 2004, Zeidner et al., 1999). Recently, SAAG-4 has been reported to be an *Ae. aegypti* salivary protein factor that can program Th_2 effector CD4 T-cell differentiation in a mouse model (Boppana et al., 2009). This polarization of host immunity towards a Th_2 response is not only beneficial to the success of blood feeding, but may also has an advantageous impact on pathogen transmission (Fontaine et al., 2011).

Together, from these information, downregulation of inflammatory (Th_1) cytokines and upregulation of anti-inflammatory cytokines (Th_2) provide a favorable environment leading to higher viral perpetuation in the skin.

5.3 Role of mosquito saliva on arboviruses infection

Mosquito borne pathogens enhance their infectivity in vertebrate host by taking advantage of the immunomodulatory properties of mosquito salivary proteins (Schneider and Higgs, 2008). Mosquito saliva impairs skin resident cells such as dendritic cells, macrophages, mast cells and neutrophils, these cells secrete chemotactic factors that help to recruit T-lymphocytes, which further mediate host defense.

Various studies demonstrated the role of mosquito saliva in flavivirus infection. In the case of WNV, mosquito saliva was reported to exacerbate the severity of pathogenesis defined as higher viremia, higher tissue titers and faster neuroinvasion and up to increase mortality rate in mouse model (Schneider et al., 2007, Schneider et al., 2006, Styer et al., 2011). Two individual studies reported a similar effects of saliva on DENV, mice had higher and sustained viremia when infected with virus transmitted by *Ae. aegypti* compared to mice infected by virus injection (Conway et al., 2014, Cox et al., 2012). Recently, Schmid and colleagues found that simutaneous inoculation of DENV with mosquito salivary gland extract (SGE) accelerates dengue pathogenesis in mice (Schmid et al., 2016). SGE directly disrupts endothelial barrier function in vitro and induces endothelial permeability in vivo in the skin. A functional genomic and proteomic analysis of salivary glands of *Ae. aegypti* mosquitoes, identified proteins that significantly enhance DENV replication (Surasombatpattana et al., 2014). Conway and colleagues demontrated that a mosquito saliva serine protease CLIPA3 enhances DENV dissemination in a mouse model. While, some saliva proteins, such as aegyptin 30 kDa, cecropin 3.8 kDa and D7 have been reported to reduce DENV replication (Conway et al., 2016, Wichit et al., 2016).

In regard to CHIKV, initial study reported that host cells cutaneous cytokines were polalized in mice bitten by infected mosquitoes (Thangamani et al., 2010). Similarly, Agarwal et al., documented that the presence of saliva during CHIKV infection of mice was found to significantly down-regulated various inflammatory genes such as TLR-3, IL-2, IFN-γ, TNF-α and up-regulated anti-inflammatory genes including IL-4 and IL-10. The authors suggested that these early events might have been responsible for increased dissemination of CHIKV to serum and peripheral organs (Agarwal et al., 2016).

However, both studies used mouse model which do not mimic the natural infection of human hosts. The effects of mosquito saliva in CHIKV infection on human skin model needs to be investigated.

These data highlight the impotance of mosquito saliva in the transmission of arboviruses. The number of salivary proteins so far characterized (Patramool et al., 2012), in various hematophagous arthropods, give a global overview of their complexity as well as their diversity both at their molecular level as well as that of their targets. It is interesting to note that only a minority of these salivary proteins has been assigned a precise function (Fontaine et al., 2011). A better understanding of the pharmacology of arthropod salivary proteins might, thus, lead to the discovery of novel vasodilator, anti-platelet, anti-clotting, analgesic or immune-modulatory compounds, used by hematophagous arthropods to counteract host defenses (Fontaine et al., 2011). In addition, the identification of molecules that mediate infectivity enhancement could allow for the production of vector-based vaccines and therapeutics that will target arthropod saliva components and interfere with viral transmission.

6. RESEARCH OBJECTIVES

The main objective of this thesis is «To investigate the role of Cholesterol, SAMHD1 protein and *Aedes aegypti* saliva on Chikungunya virus infection in human skin fibroblasts».

The specific objectives are:

- To evaluate the antiviral potential of a FDA approved drug, imipramine, via interfering with intracellular cholesterol transport on CHIKV replication in human skin fibroblasts
- To evaluate the role of host proteins involved in CHIKV and ZIKV replication in human skin fibroblasts
- To investigate the impact of *Aedes aegypti* saliva on CHIKV infection in human skin fibroblasts

CHAPTER II:
IMIPRAMINE INHIBITS CHIKUNGUNYA VIRUS REPLICATION IN HUMAN SKIN FIBROBLASTS THROUGH INTERFERENCE WITH INTRACELLULAR CHOLESTEROL TRAFFICKING

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Imipramine Inhibits Chikungunya Virus Replication in Human Skin Fibroblasts through Interference with Intracellular Cholesterol Trafficking

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Abstract

Chikungunya virus (CHIKV) is an emerging arbovirus of the *Togaviridae* family that poses a present worldwide threat to human in the absence of any licensed vaccine or antiviral treatment to control viral infection. Here, we show that compounds interfering with intracellular cholesterol transport have the capacity to inhibit CHIKV replication in human skin fibroblasts, a major viral entry site in the human host. Pretreatment of these cells with the class II cationic amphiphilic compound U18666A, or treatment with the FDA-approved antidepressant drug imipramine resulted in a near total inhibition of viral replication and production at the highest concentration used without any cytotoxic effects. Imipramine was found to affect both the fusion and replication steps of the viral life cycle. The key contribution of cholesterol availability to the CHIKV life cycle was validated further by the use of fibroblasts from Niemann-Pick type C (NPC) patients in which the virus was unable to replicate. Interestingly, imipramine also strongly inhibited the replication of several *Flaviviridae* family members, including Zika, West Nile and Dengue virus. Together, these data show that this compound is a potential drug candidate for anti-arboviral treatment.

Introduction

Chikungunya virus (CHIKV) is an arbovirus transmitted by the genus *Aedes spp*. that causes chikungunya fever, an infectious disease characterized by myalgia, joint pain, rash, and intense asthenia disease[1, 2]. A common long-term complication in many patients is severe, long-lasting, debilitating arthralgia that may persist for months or even years after infection, giving sense to the name Chikungunya, meaning "the disease that bends up the joints", coined by the Makonde people in Tanzania, where the virus was first recognized in 1952[3, 4]. Whereas initially only sporadic outbreaks of chikungunya fever have been reported on the African continent and in Southeast Asia, the spread and propagation of the vector, in particular *Aedes albopictus*, over the past decade has resulted in millions of infections in more than 50 countries, not only in tropical, but also temperate regions[5, 6]. Currently, there is no licensed vaccine or antiviral treatment available to prevent or treat CHIKV infection and this arbovirus thus clearly presents a global threat for public health.

CHIKV has icosahedral-shaped enveloped virions (approximately 70 nm in diameter) and belongs to the alphavirus genus within the *Togaviridae* family[7, 8]. Its genome is composed of a single positive stranded RNA molecule of 12 kb, organized in two open reading frames (ORFs). The 5'-terminal ORF encodes four nonstructural proteins (nsP1-4) that form a replication complex involved in the synthesis of genomic RNA, as well as a subgenomic RNA[9]. The latter is translated, via the second ORF, into six structural proteins (capsid (C) protein, E1, E2, and E3 glycoproteins and 6K/TF proteins) that are all involved in the assembly of viral particles, their attachment and entry into target cells [10-12].

CHIKV is internalized into human target cells as a result of receptor-mediated endocytosis that involves both clathrin-dependent and independent mechanisms[13, 14] and is subsequently delivered to early endosomes from which the virus capsid is released into the cytosol through the formation of fusion pores. The latter process is triggered by the low-pH environment in the endosome that induces an irreversible change in glycoprotein conformation and a dissociation of the E2/E1 heterodimers, followed by E1 trimerizations[11]. The viral RNA synthesis occurs in replication complexes located in proximity of plasma membrane[15]. The E1 and E2 proteins are translated at the endoplasmic reticulum, transported through the Golgi, processed in the trans Golgi network, and finally transported to the plasma membrane where virus budding occurs[16].

Since lipid membranes do not mix spontaneously, the fusion and budding process is not only energy-dependent, but also conditioned by the lipid composition of both the viral envelope and host cell membrane, in particular by the presence of cholesterol and sphingolipids. Therefore, abnormalities in cholesterol metabolism may affect the rate and efficiency of the production of CHIKV virions at multiple steps. Indeed, depletion of cholesterol in competent cells using methyl β-cyclodextrin was reported to strongly inhibit CHIKV infection[14], which is in line with the generally accepted idea that cholesterol is required for optimal cell membrane fusion, viral replication and budding of prototype alphaviruses, such as Sindbis virus[17] and CHIKV[18], as well as other arboviruses, including West Nile virus (WNV), Japanese encephalitis virus and dengue virus (DENV)[19-22].

Cholesterol is an important biological molecule in membrane structures and is acquired by mammalian cells via *de novo* synthesis, as well as from dietary sources by receptor-mediated endocytosis. Both synthesized and dietary cholesterol are transported through the circulation in low density lipoproteins (LDLs). This is also the case for cholesteryl esters, the form in which cholesterol is stored in cells. The LDLs are then delivered to the late endosomal and lysosomal (LE/Ls) compartments, followed by the hydrolyzation of cholesteryl esters by acid lipase and the subsequent release of free cholesterol into the cytoplasm. Unesterified cholesterol exits the LE/Ls through a mechanism that is dependent on the activity of the Niemann-Pick C (NPC) type 1 and type 2 proteins, well-known for their role in the intracellular trafficking of cholesterol[23, 24]. Their importance is underscored by the observation that mutations in either one of the NPC1 or NPC2 genes may lead to loss of function of these two endosomal membrane proteins, resulting in the development of NPC disease, a rare, but fatal, autosomal recessive, lysosomal storage disorder, characterized by an accumulation of cholesterol, sphingomyelin, sphingosine, as well as the GM1 and GM3 gangliosides within the LE/Ls[25].

The molecular phenotype of NPC disease can be mimicked by the treatment of cells with the class II cationic amphiphilic compounds U18666A (3-β-[2-(diethylamine)ethoxy] androst-5-en-17-one) or imipramine[26, 27] that block the exit of cholesterol from the LE compartment and its subsequent transfer to the cell membrane. U18666A also inhibits cholesterol biosynthesis by inhibiting oxidosqualene cyclase and desmosterol reductase[28, 29].

In the present study, we have investigated the capacity of two class II cationic amphiphilic drugs to inhibit CHIKV replication, through the interruption of cholesterol trafficking, in primary human epidermal fibroblasts, an important constituent of the skin, which is considered to be the primary entry site of the virus. The results demonstrate the therapeutic potential of such drugs.

Results

Inhibition of cholesterol trafficking inhibits CHIKV replication in human skin fibroblasts

Cholesterol has been shown to play an important role in viral infection of permissive cells. In order to examine whether CHIKV replication was dependent on the accumulation of intracellular cholesterol, the human skin fibroblast cell line HFF1 was pre-treated with vehicle or U18666A for 24 h before infection with CHIKV La Réunion strain. Treatment of HFF1 cells with increasing concentrations of U18666A resulted in a dose-dependent accumulation of intracellular cholesterol in the LE/Ls compartment, as demonstrated by 100% of colocalization of the fluorescent, high affinity cholesterol-binding, chemical filipin and the LE/Ls marker LAMP-1 (Fig. 1a, b). U18666A did not have any cytotoxic effects, even at the highest concentrations used (Supplementary Fig.S1a). Pretreatment of the cells for 24 h with U18666A to allow the accumulation of cholesterol, prior to infection by CHIKV La Réunion strain for 24 and 48 h, resulted in a dose-dependent inhibition of viral replication. The effect of drug is more pronounced at the 48 h time point, as shown by a decrease in viral RNA copy numbers in the infected cells, reaching close to 97.75% at the highest concentration of the compound (Fig. 1c and Supplementary Fig.S2a). Coherently, a dose-dependent decrease in the production of viral particles was also observed (Fig. 1d Supplementary Fig.S2b).

Imipramine, a cationic hydrophobic amine, induces, like U18666A, lysosomal accumulation of numerous lipid species, including cholesterol[30]. Because of its general clinical use in humans for more than 60 years[31], this FDA-approved, antidepressant drug was chosen to further investigate the consequences of perturbation of intracellular cholesterol trafficking on the replication of CHIKV. Imipramine was first evaluated for its capacity to induce a lysosomal lipid storage disease phenotype. Similar to U18666A, treatment of HFF1 cells with increasing doses of imipramine had no effect on the viability of the cells (Supplementary Fig.S1b) and also induced a dose-dependent intracellular

accumulation of cholesterol starting at 25 µM (Fig. 2a). Imipramine treatment, at a concentration of 75 µM, was also found to result in 100% of colocalization of filipin and LAMP-1 in the LE/Ls compartment (Fig. 2b). Next, the effect of imipramine on the replication of CHIKV belonging to the East/Central/South African genotype (La Réunion isolate) was analyzed. Treatment of HFF1 cells with imipramine at concentrations ranging from 0 to 100 µM, from 2h prior to infection to the end point of the experiment resulted in a dose-dependent decrease of numbers of CHIKV infected cells (Fig. 2d). Detectable inhibition was observed already at 10 µM whereas a complete inhibition of replication was observed at a concentration of 100 µM of imipramine (Fig. 2e and Supplementary Fig.S3a). The dose-dependent reduction in the release of infectious CHIKV particles was furthermore confirmed by plaque assay (Fig. 2f and Supplementary Fig.S3b). Finally, using Western blotting analysis the presence of nsP1, as well as nsP2 and nsP3, was no longer detectable at concentrations of ≥ 25 and ≥ 50 µM imipramine, respectively (Fig. 2c). Taken together, these data demonstrate that both U18666A and imipramine, two drugs that redirect and sequestrate cholesterol to the LE/Ls compartment, inhibit the replication of various CHIKV isolates in human skin fibroblasts.

Imipramine inhibits both fusion and RNA replication steps following CHIKV infection of human skin fibroblasts

In order to determine at which step during the viral cycle imipramine exerts its antiviral activity, its effects on the fusion and RNA replication steps of CHIKV infection were evaluated. HFF1 cells pre-treated for 24h with imipramine were transduced with CHIKV envelope-pseudotyped HIV-like particles (VLP)[32] permitting to specifically evaluate CHIKV fusion events. At a concentration of 75 µM, imipramine inhibited the capacity of the CHIKV-VLPs to transduce HFF1 cells by ~85%. Vesicular Stomatitis Virus (VSV) G glycoprotein pseudotyped particles, transduced in these cells and used as a positive control, were also sensitive to imipramine inhibition albeit this effect was significantly lower than that observed for CHIKV-pseudotypes with a reduction of about 65% (Fig. 3a). Because cholesterol perturbators have no effect on early HIV replication events, imipramine therefore likely inhibits the CHIKV fusion/entry step[33].

The effect of imipramine on the post-entry step of CHIKV infection was tested in the Huh7-CHIKV replicon cell line that enables to specifically measure viral RNA replication. Imipramine did not have any cytotoxic effects, even at the highest concentrations used (Supplementary Fig.S1c). The results show a dose-dependent decrease in *RLuc* activity which is directly proportional to CHIKV replication, with a maximal inhibition observed at doses of imipramine exceeding 75 μM (Fig. 3b). RT-PCR analysis confirmed that this was because of (due to a) reduction in viral RNA copy numbers (Fig. 3c). Moreover, cholesterol was found to colocalize with LAMP-1 in this cell line in the presence of imipramine (Supplementary Fig.S1d).

The effect of imipramine on CHIKV replication was further analyzed using a time-of-addition assay (Fig. 3d); this experiment was carried out in HFF-1 cells using high (10) MOI infection with the La Réunion isolate of CHIKV. Under all conditions imipramine caused a concentration-dependent inhibition of CHIKV RNA replication and virion production (Supplemental Fig.S4, S5). Pre-treatment of cells with imipramine for 2 h caused clear, albeit relatively mild, reduction of CHIKV replication (Fig. 3e, f). Accordingly, inhibition achieved following a 2 h pre-treatment was somewhat more prominent than that observed in the absence of pre-treatment (Fig. 3e, f), thus indicating that imipramine affected the early stages of CHIKV infection. Importantly however, inhibition was reduced, but not completely lost, if imipramine was added as late as 6 hpi (Fig. 3e, f). Together, these data demonstrate that imipramine exerts its inhibitory effects at least two different stages of the CHIKV infection cycle.

NPC proteins are crucial for CHIKV replication

Nieman-Pick type C disease, a hereditary neurovisceral disorder, impairs egress of cholesterol from the LE/Ls compartment resulting in intracellular cholesterol accumulation[34]. As U18666A and imipramine mimic a NPC-deficient phenotype, we followed an alternative approach to highlight the consequence of intracellular cholesterol accumulation on CHIKV replication, using human fibroblasts deficient in NPC1 or NPC2 proteins. As expected, NPC-deficient fibroblasts showed an intracellular accumulation of cholesterol, in contrast to fibroblasts obtained from a healthy donor, as revealed by staining with filipin (Fig. 4a). Moreover, filipin staining colocalized with that of LAMP-1 (100%

amount of colocalization), demonstrating that cholesterol accumulated specifically in the LE/Ls compartment of these cells (Fig. 4b). Absence of NPC proteins resulted in a strong decrease of CHIKV La Réunion-infection, as shown by RT-qPCR and flow cytometry (Fig. 4c,d). These results were confirmed by the observation that all NPC1- and NPC2-deficient primary fibroblasts displayed reduced infection with CHIKV La Réunion (Fig. 4e). In accordance with the results obtained with normal skin fibroblasts treated with imipramine, NPC1- and NPC2-deficient primary fibroblasts transduced with CHIKV-pseudotyped particles were impaired for viral fusion (Fig 4f). A similar decrease was observed when the cells were transduced with retroviral particles pseudotyped with VSV-G, but not with GALV-envelope glycoprotein (Fig 4g). The difference correlates with the mode of entry: VSV and CHIKV both enter into their target cells using endocytosis followed by fusion with endosomal membranes while GALV enters by fusion directly at the plasma membrane. These results show that NPC1 or NPC2 deficiency has a strong impact on the entry and/or fusion steps in the CHIKV life cycle.

Imipramine also exerts antiviral activity against Flaviviruses

In order to investigate whether the antiviral effects of imipramine are limited to CHIKV, we extended the evaluation to other arboviruses. In keeping with its antiviral effect on CHIKV, imipramine strongly inhibited, in a dose-dependent manner, the replication of WNV and DENV in human skin fibroblasts as well. It was also active against ZIKV, another member of genus Flavivirus that poses a serious global public health concern. RNA replication and virion production of each virus was effectively inhibited and approached 100% at a concentration of 100 μM (Fig. 5a-c and 5d-f, respectively and Supplementary Fig.S6), without any deleterious effects on cell viability.

Discussion

The rising global incidence of arboviruses and the absence of adequate antiviral treatment or licensed vaccines underscore the need for the development of novel and effective antiviral compounds. In the present study, we have evaluated whether drugs that interfere with intracellular cholesterol transport have the capacity to inhibit CHIKV replication in epidermal fibroblasts, a major target cell for viral

entry in the human host. The results show an antiviral activity of the class II cationic amphiphilic compounds U18666A and imipramine. Both drugs, that induce a phenotype in human fibroblasts reminiscent to that observed in Niemann-Pick type C disease, strongly inhibit the replication not only of CHIKV, but also of several members of the *Flaviviridae* family, including ZIKV, WNV and DENV without any secondary cytotoxic effects.

Cholesterol plays an important role at multiple stages during the infection cycle of a variety of viruses. It has been reported that depletion of cellular cholesterol in vitro by methyl-β-cyclodextrine significantly reduces the entry and/or fusion of a broad range of RNA viruses, including members of the Filovirus, Alphavirus and Flavivirus genera [14, 21, 22, 35] and to some extent of VSV[36, 37]. In addition, various compounds that block the cellular trafficking of cholesterol in the LE/Ls compartment, the site of viral membrane fusion and cytoplasmic escape, have been used to inhibit viral replication in permissive cells. Hence, U18666A was reported to inhibit the entry of DENV and Ebola virus[38-40] and to block assembly of HIV-1 particles[33, 40].Our results corroborate these findings and show that U18666A strongly inhibits, in a dose-dependent manner, the replication of CHIKV in human fibroblast cells. This effect is similar to that of imipramine, an FDA-approved antidepressant drug[41] and is consistent with the results from a previous study showing that imipramine inhibits the production of the Ebola virus in human umbilical vein epithelial cells[35].

In the present study, imipramine inhibited the entry and/or fusion of retroviral pseudoparticles containing the CHIKV envelope and impaired the post-fusion viral RNA replication steps, as demonstrated by the use of a stable replicon cell line and time-of-addition experiments. The drug therefore seems to interfere with distinct steps of the infectious cycle requiring cholesterol. Indeed, host membrane cholesterol is generally recognized as a key factor for the unmasking of the fusion peptide in class II envelope glycoproteins[42] including that of CHIKV[14, 43]. Moreover, as intracellular steps of alphavirus replication take place in close association with host membranes, its remains possible that cholesterol may also facilitate CHIKV RNA replication in a way that however remains to be determined.

Like other tricyclic antidepressants, imipramine undergoes biotransformation in the liver, producing progressively more polar metabolites which can then be readily excreted by the kidneys and only a

part of imipramine is eliminated without biochemical changes[44]. Pharmacologically active metabolites of imipramine are formed by N -demethylation to desipramine and hydroxylation to 2-OH-imipramine and 2-OH-desipramine, respectively, that are catalyzed by the P450 cytochrome enzymes in the liver. In the present study, we have limited ourselves to the study of the *in vitro* effects of imipramine on skin fibroblasts. However, a study in the literature reports that desipramine blocks cholesterol transport in endosomal membranes showing that this drug, like non-metabolized imipramine, also has a deleterious effect on viral trafficking and replication[45].

The antiviral effects of imipramine were also conserved for different arboviruses, including the DENV, WNV and ZIKV from genus Flavivirus, thereby attesting for its broad-spectrum antiviral activity. It is expected that these antiviral effects, in addition to a possible direct effect on viral replication, rely on the inhibition of viral fusion with cell membranes as well. Indeed, the structure of the E1 envelope glycoprotein that mediates viral fusion in Alphaviruses is very similar to its counterpart in Flaviviruses, known as Class II viral fusion proteins[42, 46]. Consequently, optimal fusion reactions of DENV and WNV also require cellular cholesterol[21, 22], accounting for their susceptibility to class II cationic amphiphilic drugs. It is of note that the requirement of cholesterol for the ZIKV life cycle has never been studied thus far, but our results provide a preliminary clue to further explore this pathway.

To further investigate the link between CHIKV infection and intracellular cholesterol trafficking, we used primary fibroblasts from patients with NPC-disease, a lysosomal storage disorder, characterized by an accumulation of cholesterol and other lipids within the LE/Ls. CHIKV infection of these cells, bearing various mutations in the NPC-1 or NPC-2 proteins, resulted in greatly reduced numbers of virus infected cells and diminished virus production. Moreover, transduction of these cells with CHIKV-glycoproteins pseudotyped particles clearly demonstrated that both NPC-1 and NPC-2-deficiency affect the entry/fusion steps in the CHIKV life cycle. The same apparently applies to VSV; in contrast transduction by GALV-pseudotyped particles that fuse directly with the plasma membrane was not affected by NPC-1 deficiency. These findings underscore the notion that NPC-1 acts in a cooperative manner with NPC-2 to traffic cholesterol within LE/Ls[23]. It is as yet unclear how the loss of function of NPC-1 or NPC-2 impacts on viral fusion. Results from several studies have shown that

NPC-1 plays a crucial role in filovirus and HIV-1 replication[33, 39]. Indeed, the presence of this protein was shown to be required for membrane fusion mediated by filovirus glycoproteins and viral escape from the vesicular compartment, independent of its known function in cholesterol transport[39].

It is important to note that cholesterol accumulation may also indirectly affect virus infection, for example by modulating receptor expression or up- or down regulation of the production of proteins with anti- or proviral activity. Other modes or action might be via a change in the composition of membranes where CHIKV RNA replication complexes are formed or where complexes of CHIKV E-proteins are assembled. Finally, higher cholesterol content may also affect the properties of progeny virions themselves, leading to the production of less or even non-infectious viral particles.

Conclusion

Although the exact steps in the CHIKV replication process affected by imipramine remains to be identified, the data presented here indicate that this kind of compounds could be effective, both at preventing infection of new target cells, as well as at decreasing the replication efficiency in cells already infected by the virus. Its possible use in antiviral treatment in infected patients however is conditioned by several limitations, such as potential side effects and the achievement of an effective dose of the drug in *in vivo* conditions. The limits of the results pertaining to the clinical use of imipramine in antiviral treatment notwithstanding, the present study describes a strategy to help design new antiviral compounds that interfere with cholesterol transport for the treatment of CHIKV and other arbovirus infections.

Materials and Methods

Cells and virus

C6/36 *Ae. albopictus* cells, used for propagation of the CHIKV strains, were grown at 28°C in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Cergy Pontoise, France) supplemented with 10% fetal calf serum (FCS; Lonza, Basel, Switzerland) at 28°C, as previously described[47]. The HFF1 skin fibroblast cell line (ATCC, Manassas, VA) and Vero cells (African green monkey kidney-derived cells, provided by P. Desprès from the Pasteur institute of Paris) were maintained in DMEM

supplemented with 15% and 5% FCS, respectively. Fibroblasts from healthy donors (GM09503, GM00500) as well as from patients carrying mutations in the NPC1 (GM00100 (1A), GM17921 (1B), GM23162 (1C), GM18397 (1D)) or NPC2 (GM18455 (2A), GM17910 (2B), GM18429 (2C), GM18424 (2D)) genes were purchased from Coriell Repositories (Coriell Institute for Medical Research, Camden, NJ). They were maintained at 37°C, 5% CO_2 in DMEM supplemented with 5% FCS. The CHIKV replicon cells were cultivated in DMEM (Life Technologies, Saint-Aubain, France) supplemented with 10% FCS, 1% penicillin-streptomycin, 1% of non essential amino acids (Life Technologies) and incubated at 37°C with 5% CO_2.

The low-passage-number of the LR2006_OPY1 strain (a kind gift from Dr Philippe Desprès, PIMIT, Inserm U1187, St Clotilde), was isolated from a viremic patient in La Réunion Island in 2006. The DENV-2 strain 16681[48] and the Israeli WNV strain IS-98-ST1 (WNV_{IS98})[49] were used in this study. The clinical isolate PF-25013-18 of ZIKV has been previously described[47]. All viruses were grown in C6/36 cells.

Antibodies and reagents

The high specificity rabbit anti-CHIKV nsP1, nsP2 and nsP3 antisera, prepared in-house[50] and previously used for detection of CHIKV ns-proteins upon use of virus inhibitors[51] were used for the detection of CHIKV nonstructural proteins. Mouse anti-CHIKV envelope 3E4 Cy3- or Alexa Fluor 488-conjugated antibodies were kindly provided by Dr Philippe Desprès. Rabbit anti-NPC1, NPC2 and LAMP-1 antibodies were purchased from ABCAM (Paris, France). Secondary antibodies (HRP conjugated goat anti-mouse or -rabbit) were purchased from Jackson Laboratories (Westgrove, PA). Mouse monoclonal anti-β –actin antibody, U18666A (3 β-[2-(diethylamino)ethoxy]an- drost-5-en-17-one), imipramine hydrochloride, filipin and thiazolyl blue tetrazolium bromide (MTT) were from Sigma-Aldrich (St Louis, MO).

Viability assay

Cell viability was determined using MTT-based assay. Briefly, cells were treated with different compounds and incubated at 37°C, 5% CO_2, washed by phosphate-buffered saline (PBS) and then

incubated with 100 µL MTT. After 2 h, MTT was removed and 50 µL of DMSO were added to each well and mixed thoroughly. The mixture was incubated at 37°C for 10 min and cellular viability was determined measuring the absorbance value at 570 nm.

Viral Infection

Human fibroblasts were seeded in six-well plates and grown to a 70-80% confluence. The cultures were rinsed twice with PBS and the cells were incubated with CHIKV, DENV, WNV and ZIKV at the desired MOI for 2 h at 37°C while gently agitating the plates. Then, the inoculum was removed and the cells were washed three times with PBS. DMEM supplemented with 15% FCS was added to each well and the plates were incubated at 37° C and 5% CO_2, for the duration of the experiment. Cells serving as a negative control were incubated with culture supernatant from uninfected C6/36 cells.

CHIKV replicon cell line based assay

Huh-7 cells stably transfected with the CHIKV La Réunion-NCT (Non Cytotoxic) replicon[52, 53] were used to test the effect of imipramine on CHIKV RNA replication. CHIKV replicon cells were plated in six-well plates, treated with increasing concentrations of imipramine and incubated at 37° C and 5% CO_2. After 48 h of incubation, Renilla Luciferase (*Rluc*) activity, expressed by the CHIKV replicon, was detected using the Renilla Luciferase assay (Promega, Charbonnière, France). The luminescence signal, proportional to the CHIKV's RNA replication, was then measured using the *Modulus microplate luminometer* (Turner BioSystems, CA) and plotted to determine the antiviral activity of imipramine. Vehicle-treated cells were used as a control. A sigmoidal curve fit with variable slope was created to obtain the half maximal effective concentration (EC_{50}) value using Graph Pad Prism 5 (Graph Pad Software Inc., San Diego, CA).

Cholesterol staining

Cells were stained by filipin as previously described[33]. Briefly, cells were fixed with paraformaldehyde (2%) for 15 min at room temperature, washed three times with PBS and incubated

with filipin for 30 min at 4°C. Cells were collected, washed three times with PBS and analyzed by fluorescence microscopy in the DAPI channel.

Cell staining and immunofluorescence assay

CHIKV-infected cells were fixed with 2% paraformaldehyde for 30 min at RT and then permeabilized with 0.1% saponin for 15 min at 4°C. The cells were incubated with the Cy3-conjugated anti-CHIKV antibody 3E4 in the presence of 0.1% saponin for 1 h at 4° C. The cells were then washed three times with PBS and stained with filipin. Stained cells were mounted onto glass slides using ProLong antifade mounting media (Molecular Probes, Eugene, OR) and fluorescence was analyzed by Leica microscope. Percentage of colocalization was analysed by JACoP (http://rsb.info.nih.gov/ij/plugins/track/jacop.html) using ImageJ program.

Plaque assay

Vero cells, grown to 70-80% confluence, were incubated with four separate, ten-fold, dilutions of viral supernatant in DMEM at 37° C for 2 h. Then, a mix of nutriment solution with agar (Lonza) was added and the cells were maintained at 37° C for 6 days. For plaque counting, the cells were incubated with 3.7% formaldehyde and 0.5% Crystal violet in 20% ethanol.

U18666A and imipramine treatment

Human fibroblast cells were pre-incubated with vehicle or increasing concentrations of imipramine or U18666A for 2 or 24 h, respectively. Then, treated cells were infected with CHIKV for 24 h or 48 h before further analysis. For imipramine treatment, the cells were maintained with the drug throughout the infection.

For time-of-addition assay cell cultures were infected with CHIKV at MOI of 10; imipramine was use at concentrations 10μM, 25μM, 50μM, 75μM or 100μM and DMSO was used as vehicle control. Cells were pre-treated for 2 h with the inhibitor that was either discarded prior to infection with CHIKV (pre-treatment only) or, alternatively, was maintained in the cell culture throughout the infection. In other setups, the compounds were added together with virus or at 6 hpi and were present

until cells were harvested at 48 hpi. The amounts of released virions were determined using plaque titration in Vero cells.

Western blotting analysis

Cells were lysed on ice in RIPA buffer (150 mM NaCl, 5 mM β-mercaptoethanol, 1% NP-40, 0.1% sodium dodecyl sulfate, 50 mM Tris-HCl, pH 8) supplemented with protease inhibitor cocktail solution (Sigma). The protein concentration was determined by bicinchoninic acid (BCA) assay (Thermo Scientific, Saint Herblain, France). Equal amounts of proteins were mixed with Laemmli sample loading buffer, heated for 5 min at 100°C, subjected to SDS-PAGE and electrotransferred onto a nitrocellulose membrane. The membrane was blocked with 0.05% Tween 20 in PBS (PBST) containing 5% skim milk for 1 h at RT, incubated overnight at 4°C with desired primary antibody, washed three times with PBST, and subsequently incubated for 1 h at RT with horseradish peroxidase-coupled secondary antibodies (Cell Signaling, France) in PBST. The membrane was washed three times, and proteins were detected by chemiluminescence using a SuperSignal West Pico chemiluminescent substrate kit (Thermo Scientific). The membrane was then stripped and re-probed with an anti-β-actin to ensure that equivalent levels of protein were loaded in each lane.

Flow cytometry analysis

Infected cells were trypsinized, washed three times in PBS and infection efficiencies were determined by flow cytometry as previously described[47]. Briefly, cells were fixed with 2% paraformaldehyde, permeabilized using 0.1% saponin, and stained with an anti-CHIKV 3E4 Alexa Fluor 488-conjugated antibody. Stained cells were analyzed using the Becton FACSCalibur flow cytometer (Cell Quest software).

Viral RNA quantification by real time RT-PCR

Total RNA was extracted from human fibroblasts by using Tri reagent (Sigma, Saint Quentin Fallavier, France). The RNA pellet was resuspended in 25 µl of RNase-free distilled water and stored at -80°C. The RNA was used for reverse transcription using Moloney murine leukemia virus (M-

MLV) reverse transcriptase (Promega, Charbonnieres, France) according to the manufacturer's instructions. The reaction was carried out using 1 µg total RNA as template for the normalization of viral RNA to the amount of total RNA. The MaximaTM Probe/ROX qPCR Master Mix (2x) (Thermo Scientific) was used in qPCR experiment. Each reaction of 25 µL contained 400 nM of each primer, 200 nM of specific probe and 1x Maxima Probe/ROX qPCR Master Mix. Primers and probes sequences are listed in Supplementary Table S1. The amplification conditions were 95 °C for 10 min followed by 45 amplification cycles of 95 °C for 15 s, 60 °C for 20 s and 72 °C for 30 s. The reactions were performed in an Applied Biosystem 7300 system. Real time data were analyzed using the SDS software (Thermo Fischer Scientific). Viral RNA was quantified by comparing the sample's threshold cycle (Ct) values with each virus RNA standard curve which was obtained as previously described[47, 54].

Production of CHIKV glycoprotein pseudotyped particles

Production of lentivirus particles pseudotyped with CHIKV La Réunion glycoproteins was achieved as previously reported[32]. Briefly, the pCAGGS-EnvCHIKV plasmid encoding 3-E1 region of CHIKV (amino acids 262 to 1248) (a kind gift from Graham Simmons, Blood Systems Research Institute, San Francisco, CA)[55] or the pCMV-MD-VSV-G and pCMV-MD-GALV plasmids encoding the vesicular stomatitis virus (VSV) glycoprotein G or the Gibon Ape Leukemia virus (GALV) envelope protein, respectively (kindly provided by Jean-Luc Battini, IGMM, UMR5235-CNRS Montpellier) were coexpressed with a GFP reporter pseudogenome in cells stably expressing the HIV-1Gag and Pol proteins. Viral pseudoparticles contained in the culture supernatant were purified by ultracentrifugation, their amounts were normalized according to HIV-1 p24 content measured by ELISA (Innogenetics). Fusion of viral pseudoparticles was monitored by quantification of GFP expression in the target cells. 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 to the protein content in the sample determined using the BCA Assay (Pierce).

Data analysis and statistical methods

All data are presented as means ± standard deviation (SD). The student *t* test was used to determine the statistical significance. For all experiments, statistical significance was accepted when at $p < 0.05$.

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Author contributions

S.W., R.H., E.B., L.T., F.D., P.F. and F.L. carried out the entire experiments and S.W., H.Y. and D.M. wrote the manuscript and P.E., N.L., P.S., A.M., V.C., P.R., L.B. and F.T. reviewed the manuscript. All authors read and approved the final version of the manuscript.

Additional information

Supplementary information:

Competing financial interests: The authors declare no competing financial interests.

Figure 1:

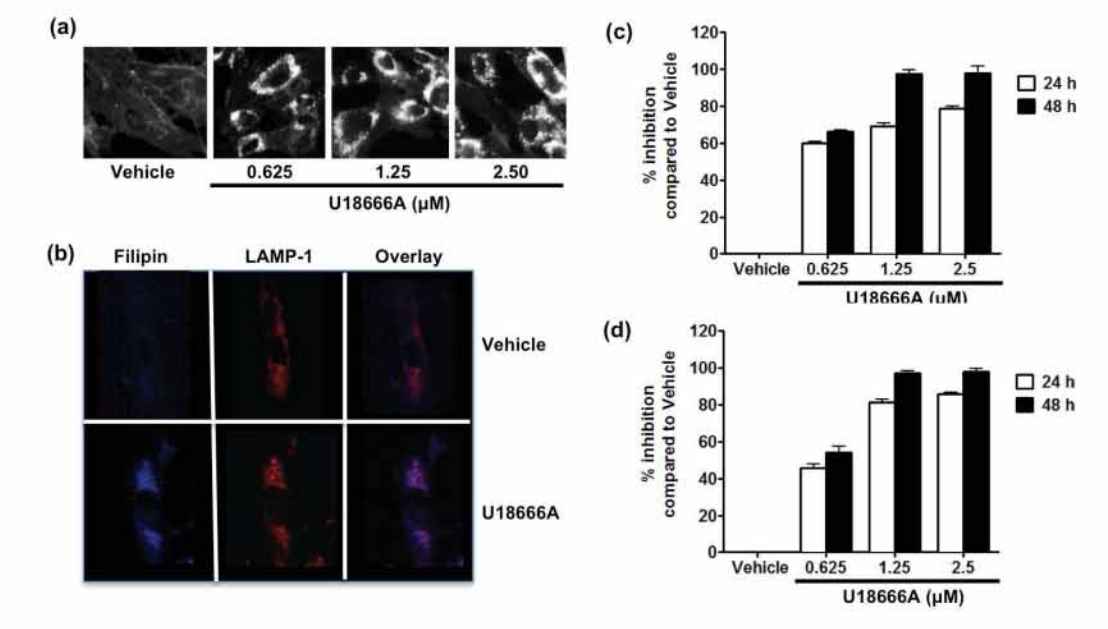

Figure 1. U18666A causes accumulation of cholesterol in the late endosome/lysosome and inhibits CHIKV replication in human skin fibroblasts (a) Human skin fibroblasts were treated either by water (vehicle) or 0.625, 1.25 and 2.5 µM of U18666A for 24 h before fixation and labeling by filipin. (b) Human skin fibroblasts were treated with vehicle or 2.5 µM of U18666A for 24 h before fixation and labeling by filipin (blue) or LAMP-1 (Red). Colocalization of cholesterol and LAMP-1 are shown in violet. (c and d) Human skin fibroblasts were treated with vehicle or U18666A for 24 h before infection with CHIKV La Réunion strain (MOI 1). After 24 and 48 h, virus RNA and infectious virus production were measured by real time RT-PCR and plaque assay, respectively. Inhibition at the presence of vehicle was set as "0" and percentage of inhibition at the presence of inhibitor was calculated using formula [1-(I/V)]*100 where V and I designate experimental values (RNA copy numbers or plaque numbers) at the presence of vehicle and inhibitor, respectively. The data represent mean ± SD from three independent experiments.

Figure 2:

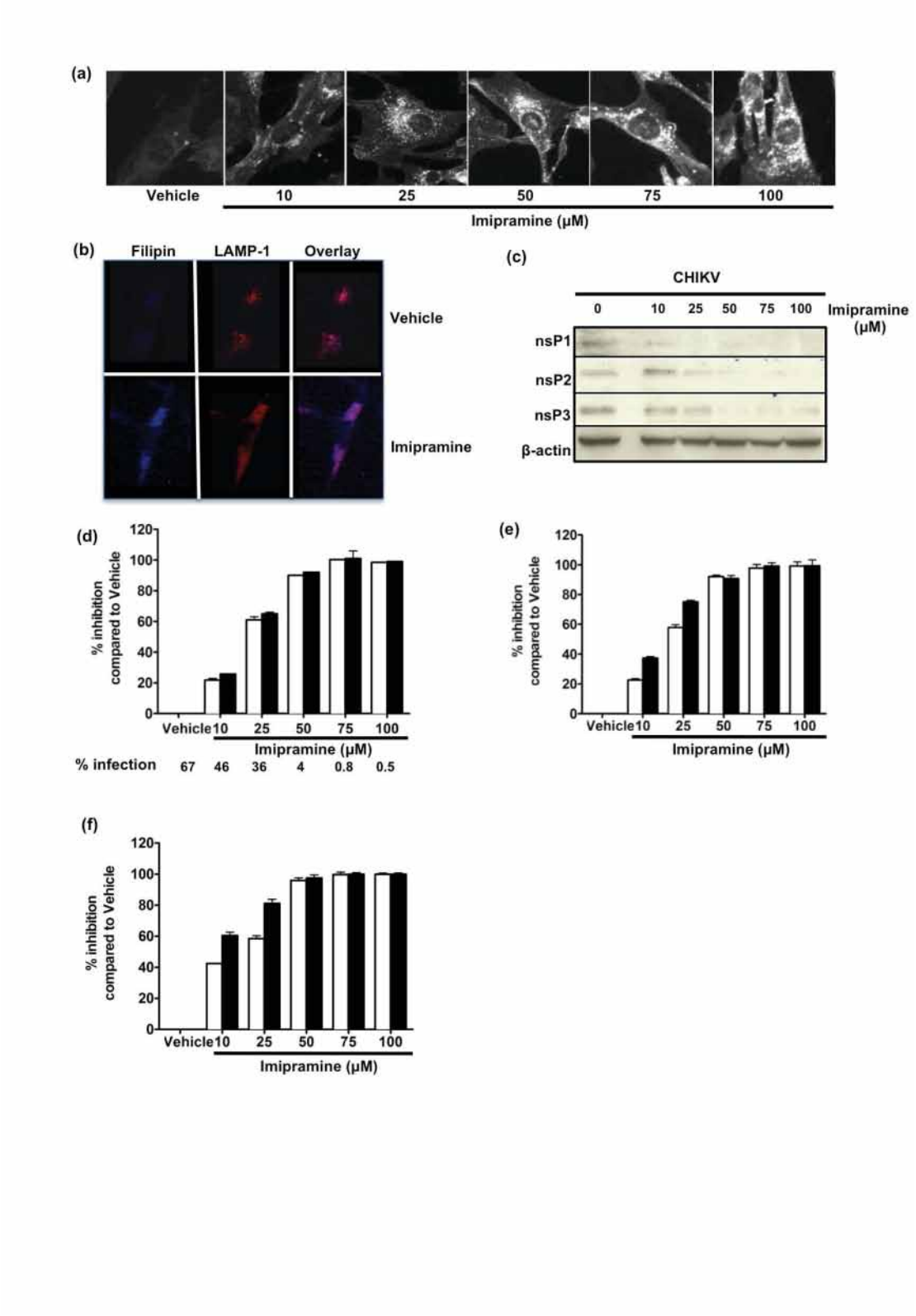
(a)
Vehicle
10
25
50
75
100
Imipramine (μM)
(b)
Filipin
LAMP-1
Overlay
Vehicle
Imipramine
(c)
CHIKV
0
10
25
50
75
100
Imipramine (μM)
nsP1
nsP2
nsP3
β-actin
(d)
% inhibition compared to Vehicle
Vehicle
10
25
50
75
100
Imipramine (μM)
% infection
67
46
36
4
0.8
0.5
(e)
% inhibition compared to Vehicle
Vehicle
10
25
50
75
100
Imipramine (μM)
(f)
% inhibition compared to Vehicle
Vehicle
10
25
50
75
100
Imipramine (μM)

Figure 2. Imipramine causes accumulation of cholesterol in the late endosome/lysosome and inhibits CHIKV replication in human skin fibroblasts (a) Human skin fibroblasts were treated either with PBS (vehicle) or 10, 25, 50, 75 and 100 μM concentrations of imipramine for 24 h before fixation and labeling by filipin (for a clear outline the color was changed to black and white). (b) Human skin fibroblasts were treated by vehicle or 75 μM of imipramine for 24 h before fixation and labeling by filipin (blue) or LAMP-1 (Red). Colocalization of cholesterol and LAMP-1 were shown in violet. (c-i) Human skin fibroblasts were pretreated by vehicle or indicated concentrations of imipramine for 2 h before exposed to CHIKV (MOI 1) La Réunion (24 h (white bar) and 48 h (black bar)) strain (imipramine maintained throughout the infection). (c) Infected cells were lysed with RIPA and analysed by immunoblotting against nsP1, nsP2, nsP3 and β-actin. The samples were derived from the same experiment and blots were processed in parallel. Reduction of percentage of CHIKV infected cells (d), inhibition of virus RNA synthesis (e) and infectious virus productions (f) were quantified by flow cytometry, real time RT-PCR and plaque assay, respectively. Percentage of inhibition was calculated as for Fig. 1. The data represent mean ± SD from three independent experiments.

Figure 3:

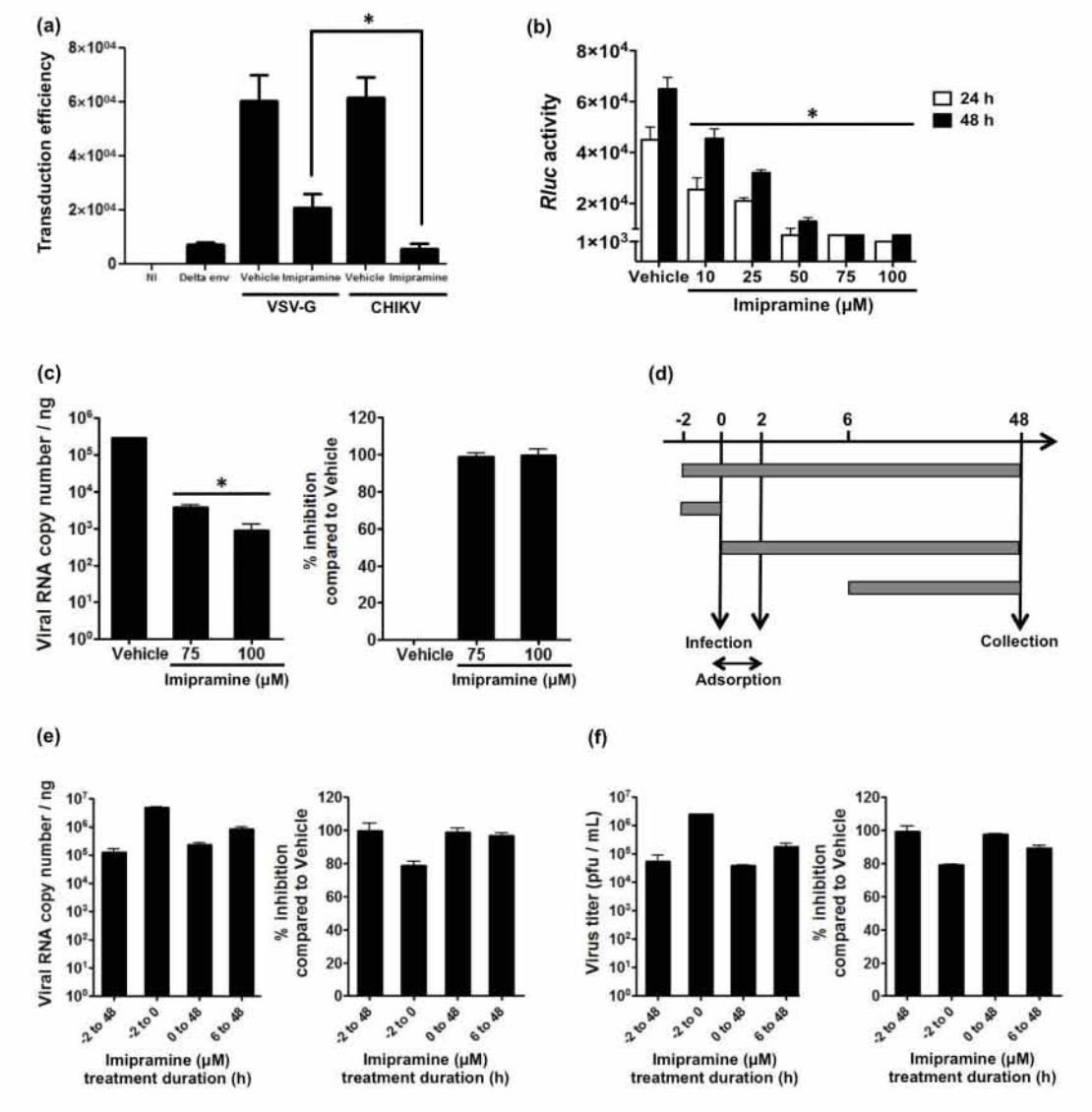

Figure 3. Imipramine exerts its inhibitory effects at different stages of the viral life cycle. (a) Human primary skin fibroblasts were either treated with 75 μM imipramine or with PBS (vehicle) for 24 h and then challenged with CHIKV Env- or VSV-G-pseudotyped HIV particles. HIV particles devoid of envelope were used to monitor potential pseudo-transduction events. Transduction of the cells was monitored by quantification of GFP expression in the cells after 48 h. Values are normalized according to protein contents of the cell extract and correspond to the mean of triplicate ± SD. NI is for non-transduced cells. *, $p < 0.05$. (b) Stable transfected Huh-7 cells with CHIKV-NCT replicon were treated with different concentrations of imipramine. After 24 and 48 h, treated cells were measured for the Renilla Luciferase (*Rluc*) activity. Vehicle–treated (PBS) cells were used as control ("0")

concentration. Relative *Rluc* activity expressed by the CHIKV replicon represents CHIKV's RNA replication. Values are normalized according to protein contents of the cell extract and correspond to the mean of triplicate ± SD. (c) Results of RT-PCR analysis of viral RNA copy numbers in vehicle and imipramine treated cells. Percentage of inhibition (right panel) is calculated as described for Fig.1. The data represent mean ± SD from three independent experiments. *, $p < 0.05$ when compared to cells treated with vehicle. (d) Schematic presentation of the time-of-addition assay. Human skin fibroblasts, treated as shown with 10 µM, 25 µM, 50 µM, 75 µM or 100 µM of imipramine, were infected with CHIKV La Réunion strain for 48 h. (e) Inhibition of virus RNA synthesis by 75 µM of imipramine was quantified using real time RT-PCR. Viral RNA copy numbers, obtained under different conditions, are shown on the left panel; right panel shows achieved percentage of inhibition calculated as for Fig.1. (f) Inhibition of infectious virus production by 75 µM of imipramine was analyzed using plaque assay. Virus titers, obtained under different conditions, are shown on the left panel; right panel shows achieved percentage of inhibition calculated as for Fig 1. Data for all used concentrations is shown on Supplemental Fig S4 and S5.

Figure 4:

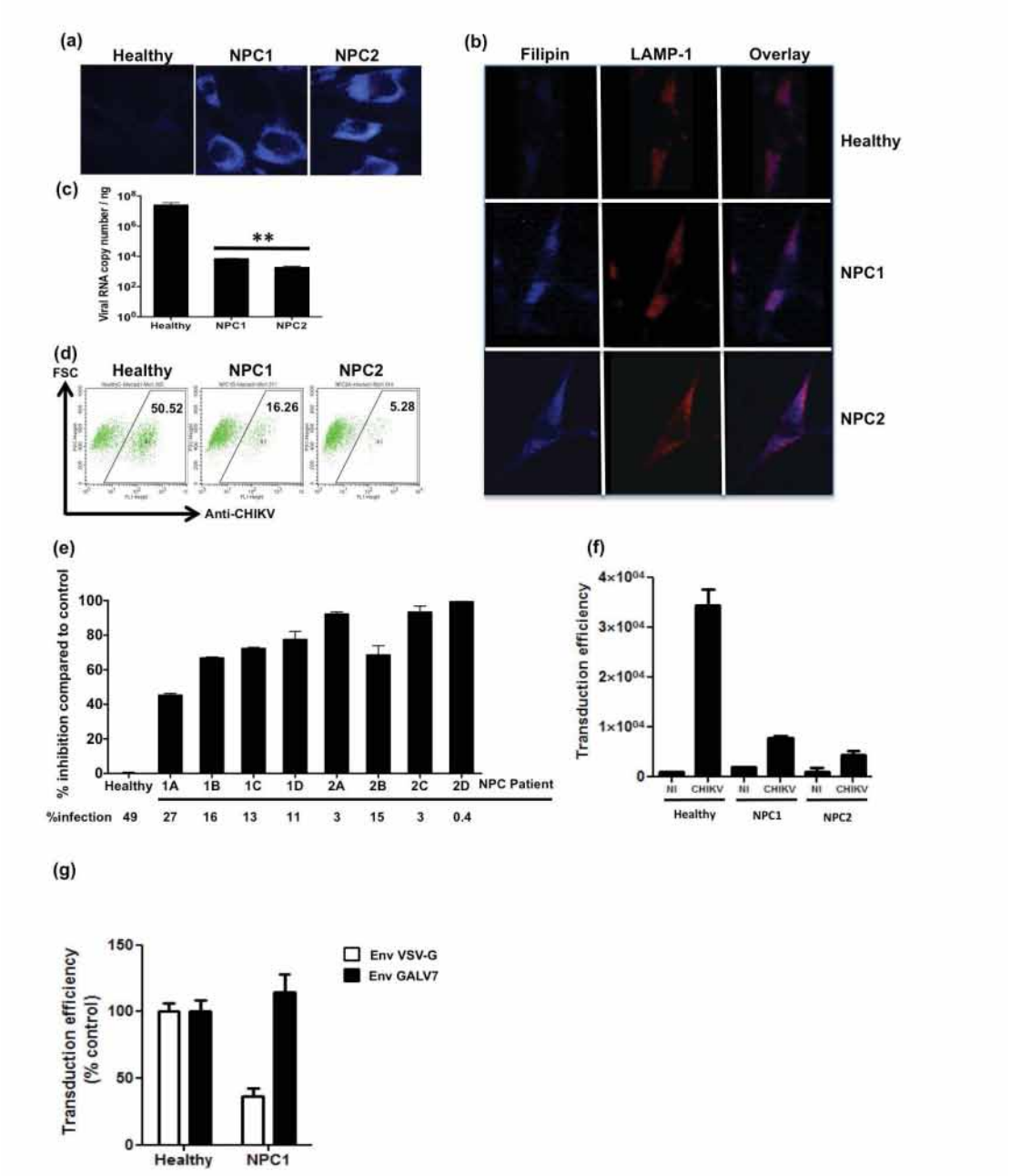

Figure 4. NPC1 and NPC2 proteins are crucial for CHIKV replication. (a) The phenotypes of NPC-deficient cells were confirmed by filipin and cholesterol accumulation examined by fluorescence microscope. (b) Healthy, NPC1- and NPC2-deficient cells were fixed and labeled with filipin (blue) or LAMP-1 (red). Colocalization of cholesterol and LAMP-1 are shown in violet. (c and d) Healthy, NPC1- and NPC2-deficient cells were infected with CHIKV La Réunion strain (MOI 1). After 24 h, the production of viral RNA and production of virus structural proteins were measured by real-time

RT-PCR and FACS analysis, respectively. The data represent mean ± SD from three independent experiments. **, $p < 0.01$ when compared to healthy. (e) Healthy, NPC1- and NPC2-deficient cells were infected with CHIKV La Réunion strain (MOI 1). After 24 h, percentage of CHIKV infected cells was measured by flow cytometry; reduction of percentage of CHIKV infected cells compared to cells from healthy control is shown. (f) Primary fibroblasts derived either from healthy individuals or from patients with NPC1 or NPC2 deficiencies were challenged with HIV particles pseudotyped either with CHIKVenvelope glycoproteins. Transduction of the cells was monitored by quantification of GFP in the cells after 48h. Values are normalized according to protein content in the samples and are the mean of triplicated ± SD. NI is for non transduced cells. (g) Normal skin fibroblasts or NPC1 deficient cells were transduced by VSV-G- or GALV-pseudotyped particles and processed as in (f).

Figure 5:

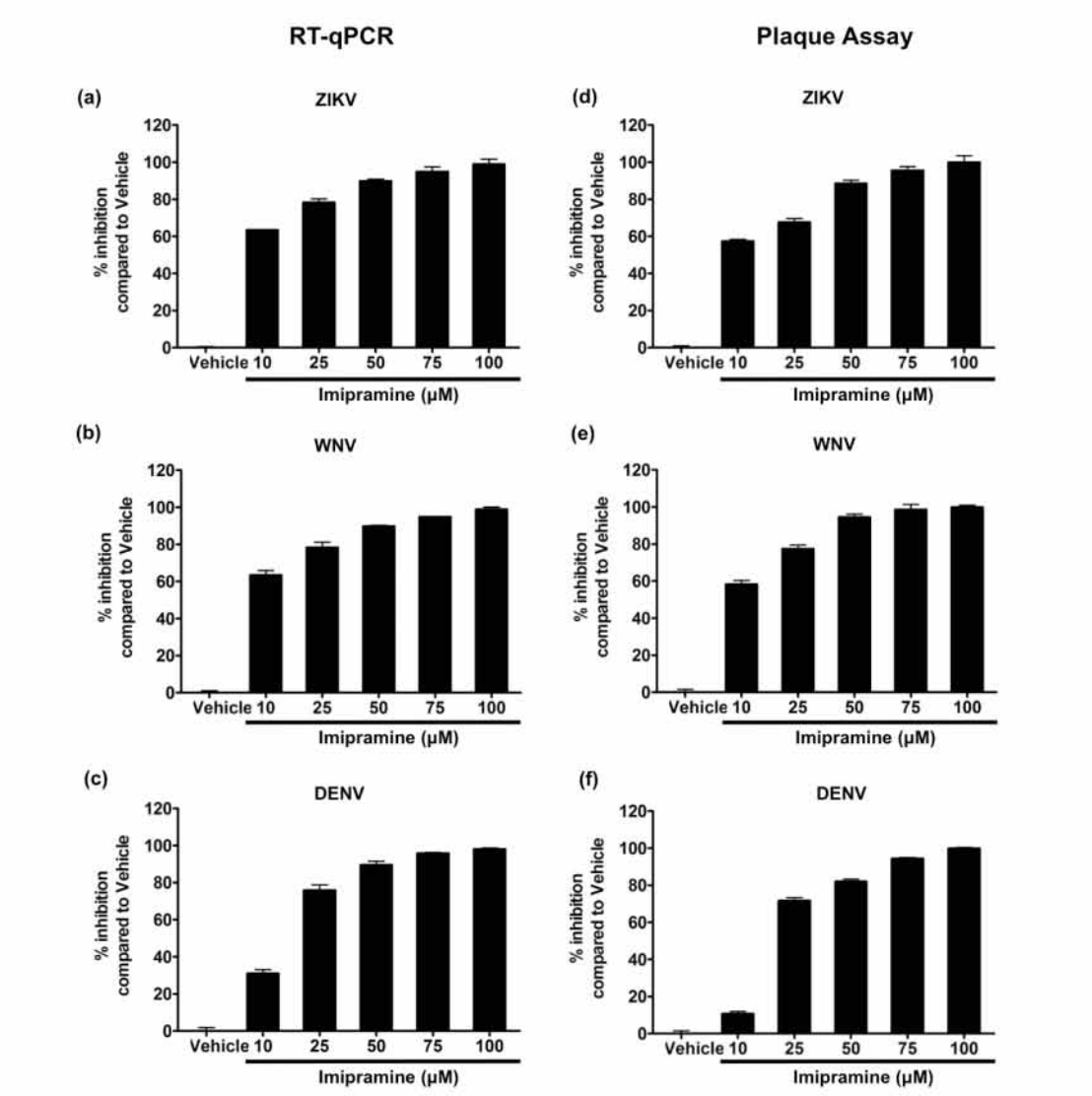

Figure 5. Imipramine inhibits Flavivirus replication in primary human skin fibroblasts. Human skin fibroblasts were pretreated with vehicle or imipramine at the indicated concentrations for 2 h before exposure to different Flaviviruses. Imipramine was present throughout the culture. After 48h of culture, the presence of virus RNA in cells and infectious virus productions were quantified by RT-PCR and plaque assay, respectively. (a and d) ZIKV strain Pf13 (MOI 0.1) (b and e) WNV WT (MOI 0.1) (c and f) DENV-2 strain 16681 (MOI 0.5). The data represent mean ± SD from three independent experiments.

CHAPTER III:
SAMHD1 ENHANCES CHIKUNGUNYA AND ZIKA VIRUS REPLICATION IN HUMAN SKIN FIBROBLASTS

Manuscript in preparation

SAMHD1 Enhances Chikungunya and Zika Virus Replication in Human Skin Fibroblasts

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Abstract

Chikungunya virus (CHIKV) and Zika virus (ZIKV) are emerging arboviruses that pose a worldwide threat to human health. Currently, neither vaccine nor antiviral treatment to control their infections is available. As the skin is a major viral entry site for arboviruses in the human host, we have, in the present study, determined the global proteomic profile of CHIKV and ZIKV-infections in human skin fibroblasts using Stable Isotope Labelling by Amino acids in Cell culture (SILAC)-based mass-spectrometry analysis. We show that interferon-stimulated proteins (MX1, IFIT1, IFIT3, ISG15) and defense response proteins (DDX58, STAT1, OAS3, EIF2AK2, SAMHD1) are significantly up-regulated in human fibroblasts infected with either virus. Ectopic expression of IFITs proteins markedly inhibited CHIKV and ZIKV replication which, accordingly, was restored following the abrogation of IFIT1 or IFIT3 in human fibroblasts. Overexpression of SAMHD1 in cutaneous cells, or pretreatment of cells with the virus-like particles containing SAMHD1 restriction factor Vpx, resulted in a strong increase or inhibition, respectively, of both CHIKV and ZIKV replication. Moreover, silencing of SAMHD1 by specific SAMHD1-siRNA resulted in a marked decrease of viral RNA levels. Type I IFN treatment increased SAMHD1 protein levels in human skin fibroblasts in a time-dependent manner. Together, these results suggest that IFITs are involved in the restriction of replication of CHIKV and ZIKV and provide, as yet unreported, evidence for a proviral role of SAMHD1 in arbovirus infection of human skin cells.

Introduction

Arbovirus infection causes an increasing global burden for public health. Although the presence of many arboviruses are geographically restricted, they can unexpectedly spread within their area of endemicity and the past five decades have seen an unprecedented emergence of epidemic arboviral diseases. In this respect, there has been a major geographical expansion of Chikungunya virus (CHIKV) and Zika virus (ZIKV) in Central and South America [1].

CHIKV is a member of the *Togaviradae* family, belonging to the genus alphavirus, that is present in tropical and subtropical regions [2]. Lately, it has spread to the Western hemisphere and is now endemic in the Caribbean islands and South and Central America, including Mexico [1]. Up to ninety percent of infected individuals develop Chikungunya fever, characterized by high fever, myalgia, joint pain, rash, and intense asthenia [3]. Frequently long-term complications such as encephalopathy, encephalitis, myocarditis, hepatitis, and circulatory failure occur [4, 5].

ZIKV belongs to the genus flavivirus of the *Flaviviridae* family [6]. The largest ZIKV pandemic occurred in Brazil in 2015 and subsequently spread throughout the Americas infecting nearly two million people [7]. The clinical manifestations of ZIKV infection range from asymptomatic to a flu-like illness. More recently, an increase in the incidence among infected adults of Guillain-Barré syndrome and neurological birth defects, including microcephaly in newborns, was observed [8] that has been substantiated by the observation that ZIKV is a teratogenic agent able to cross the placenta barrier and to inflict severe neurological damage in developing fetuses [9, 10]. Currently, neither vaccine nor specific antiviral treatment is available to prevent or treat CHIKV and ZIKV infections.

CHIKV and ZIKV are predominantly transmitted by *Aedes (Ae.) aegypti* and *Ae. albopictus* mosquitoes [11]. Human infections with arboviruses occur during blood feeding by infected mosquitoes. During blood meals, the mosquito′s mouthpieces are introduced into the skin, resulting in extravascular delivery of infectious viral particles in both the epidermis and dermis where resident and migratory cells encounter the pathogen [12, 13].

To gain more insight into the complex interactions between CHIKV and ZIKV with the skin, we determined the proteome profile of human skin fibroblasts (HFF1) during viral infection, using Stable Isotope Labeling by Amino acids in Cell culture (SILAC) based LC-MS/MS analysis. Previous studies have focused on the proteome profile on CHIKV infection and identified several differentially expressed proteins involved in the stress response, cytoskeleton/cell structure, transport/trafficking and signaling, using neuronal cells, myocytes and hepatocytes [14-17]. However, the latter cells do not represent a major entry site for CHIKV and ZIKV, unlike the skin, as we have reported recently [18, 19]. Therefore, in the present study we have analyzed the proteome changes during infection of human fibroblasts with the aim to reveal novel molecular details of the cellular processes involved in replication of these arboviruses.

Here, we report the identification of 16 differentially expressed proteins following infection of HFF1 cells with either CHIKV- or ZIKV-infected cells, including interferon (IFN)-stimulated proteins and defense response proteins. Moreover, we show that the Sterile Alpha Motif (SAM) domain and histidine/aspartic acid (HD) domain containing protein 1 (SAMHD1) favors both CHIKV and ZIKV replication, whereas IFN-induced proteins with tetratricopeptide repeats (IFITs) may play a role in its inhibition.

Materials and Methods

Human cells and viruses

C6/36 *Ae*. *albopictus* cells, used for propagation of the CHIKV and ZIKV strains, were grown at 28°C in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Cergy Pontoise, France) supplemented with 10% fetal calf serum (FCS; Lonza, Basel, Switzerland) at 28°C, as previously described [19]. The HFF1 cell line (ATCC, Manassas, VA) and Vero cells were maintained in DMEM supplemented with 15% and 5% FCS, respectively. WT- and SAMHD1-U937 stable cells have been previously described [20].

The low-passage-number of the LR2006_OPY1 strain (isolated from a viremic patient in La Réunion Island in 2006) was a kind gift from Dr Philippe Desprès (PIMIT, Inserm U1187, St Clotilde). The

clinical isolate PF-25013-18 of ZIKV has been previously described [19]. Both virus strains were grown in C6/36 cells. Lentiviral virus like particles (VLPs) containing Vpx (VLPs/Vpx) were generated as previously described [21].

Antibodies and reagents

Anti-SAMHD1 antibody was purchased from Abcam (Paris, France). Anti-phospho SAMHD1 was a prepared in house. Anti-IFIT1, 2, 3, MX1 and β-actin antibodies were purchased from GeneTex (Souffelweyersheim, France). Recombinant human alpha interferon (IFN-α), IFN-β, and IFN-γ were purchased from R&D Systems (Lille, France).

SILAC labeling and virus infection

HFF1 cells were cultured in SILAC DMEM (PAA, Austria), 10% dialyzed FBS (Gibco, UK), 0.280 mM arginine, 0.398 mM lysine, 0.5 mM proline, 10 mM HEPES, 2 mM L-glutamine and 100 IU penicillin and 100 µg/mL streptomycin for > 5 cell doublings to ensure complete incorporation of the labeled amino acids. Arginine to proline conversion was not observed under these labeling conditions. The SILAC light medium (L) was supplemented with Arg $^{12}C_6{}^{14}N_4$ and Lys $^{12}C_6{}^{14}N_2$ (Sigma, The Netherlands), the SILAC heavy medium (H) was supplemented with Arg $^{13}C_6{}^{15}N_4$ and Lys $^{13}C_6{}^{15}N_2$ (Cambridge Isotope Laboratories, Massachusetts, USA).

HFF1 cells were seeded in six-well plates and grown to a 70-80% confluence. The cultures were rinsed twice with PBS and the cells were incubated with either CHIKV or ZIKV at multiplicity of infection (MOI) of 8 for 2 or 1.5 h, respectively, at 37°C while gently agitating the plates. Then, the inoculum was removed and the cells were washed three times with PBS. SILAC DMEM supplemented with 15% FCS was added to each well and the plates were incubated at 37° C and 5% CO_2. Infected and mock-infected cells were lysed at 48 h post-infection in 4% SDS, 0.1 M Tris pH 7.6.

Mass spectrometry analysis

Proteins were separated on SDS-PAGE gels (10% polyacrylamide; Mini-Protean TGX Precast Gels; Bio-Rad) and stained with Page Blue Stain (Fermentas). Gel lanes were cut into 9 gel pieces and

destained by three washes in 50% acetonitrile and 50 mM triethylammonium bicarbonate (TEABC). After protein reduction (with 10 mM dithiothreitol in 50 mM TEABC at 56°C for 45 min) and alkylation (55 mM iodoacetamide TEABC at RT for 30 min), proteins were in-gel digested using trypsin (500 ng/band; Gold; Promega).

Peptides were analyzed online by nano-flow HPLC-nanoelectrospray ionization using a LTQ-orbitrap Elite mass spectrometer (Thermo Fisher Scientific) coupled to a nano-LC system (U3000 Dionex, Thermo Fisher Scientific). Desalting and preconcentration of samples were performed on-line on a Pepmap® precolumn (0.3 × 10 mm; Dionex). A gradient consisting of 0-40% B in A for 80 min (A: 0.1% formic acid, 2% acetonitrile in water, and B: 0.1% formic acid in acetonitrile) at 300 nl/min, was used to elute peptides from the capillary reverse-phase column (0.075 × 150 mm, Pepmap®, Dionex). Data were acquired using the Xcalibur software (version 2.2). A cycle of one full-scan mass spectrum (400-2,000 m/z) at a resolution of 120,000 (at 400 m/z), followed by 20 data-dependent MS/MS spectra (LTQ) was repeated continuously throughout the nanoLC separation. Raw data analysis was performed using the MaxQuant software (version 1.5.0.0) with standard settings [22]. Used database consist of Human, ZIKV and CHIKV entries from Uniprot and 250 contaminants (MaxQuant contaminant database). Relative proteins quantifications were calculated on the median SILAC ratios. For further analysis we consider only proteins with at least two peptides. Determination of significant proteins was assessed using significance A, using Perseus (version 1.5.0.0) on the log2 ratio after elimination of contaminant and reverse entries.

Bioinformatics analysis

We selected as significantly up-regulated proteins all those proteins having inversed ratios in H/L and L/H conditions and a detection *p*-value smaller than 0.05 in both conditions.

We used the g:Profiler tool [23] (November 2015, version: r1477_e82_eg29) to assess the over-representation of Gene Ontology and biological pathways annotations (KEGG and Reactome) among the up-regulated proteins. We considered as significant those annotations having an adjusted *p*-value <0.025 after Benjamini-Hochberg multiple testing correction. We built a human functional interaction

network among CHIKV and ZIKV up-regulated proteins by using the STRING database v10 [24]. We selected all the functional interactions having a medium confidence score (*i.e.*, equal or greater than 0.4).

VLPs/Vpx treatment

HFF1 cells were pre-incubated with vehicle or VLPs/Vpx for 24 h. Then, treated cells were infected with CHIKV or ZIKV for 2 or 1.5 h before further experiments. The cells were maintained with VLPs/Vpx throughout the infection.

Infection

Cells were grown to 70% confluence in six-well plates. At the day of infection, cells were washed twice with phosphate-buffered saline before inoculated with viruses at the desired MOI for different periods. Negative control cells were prepared by incubating the cells with culture supernatant from uninfected C6/36 cells.

Small interfering RNA (siRNA) transfection

Cells were transiently transfected with a 80 nM final concentration of small interfering RNAs (siRNAs) target SAMHD1 by using Lipofectamine® 2000 (Thermo Fisher Scientific, Massachusetts, USA). For IFITs proteins, a 25 nM final concentration of siRNAs was used. After 24 h, cells were infected by CHIKV or ZIKV. The following pools of siRNAs (ON-TARGETplus SMARTpool) used in this study were from Dharmacon: siRNA pools for SAMHD1 (L-013950-01), IFIT1 (L-019616-00), IFIT2 (L-012582-02) and IFIT3 (L-017691-00). A nontargeting pool (NT) was used as a negative control.

Generation of SAMHD1 stable cell lines through lentivirus vector

HFF1 cells were plated at a density of $5x10^4$ cells per well in 24 wells plate. After incubate at 37°C with 5% CO_2 for 24 h, Control-GFP-lentivirus and SAMHD1-GFP-lentivirus were transducted according to the manufacturer's protocol (ABM®, Richmond, Canada). Transducted cells were sort by FACSAria III (BD Biosciences, San Jose, CA) and expanded in DMEM supplemented with 15% FCS

and 2.5 μM pyromycin. Transduced cells were validated the expression by Becton FACSCalibur flow cytometer (Cell Quest software).

Western blotting analysis

Cells were lysed on ice in TETN-150 supplemented with protease inhibitor cocktail solution (Sigma) [20]. The protein concentration was determined by bicinchoninic acid (BCA) assay (Thermo Scientific, Saint Herblain, France). Equal amounts of proteins were mixed with Laemmli sample loading buffer, subjected to SDS-PAGE and electrotransferred onto a nitrocellulose membrane. The membrane was blocked with 0.05% Tween 20 in PBS (PBST) containing 5% skim milk for 1 h at RT, incubated overnight at 4°C with desired primary antibody, washed three times with PBST, and subsequently incubated for 1 h at RT with horseradish peroxidase-coupled secondary antibodies (Cell Signaling, France) in PBST. The membrane was washed three times, and proteins were detected by chemiluminescence using a SuperSignal West Pico chemiluminescent substrate kit (Thermo Scientific). The membrane was then stripped and re-probed with an anti-β-actin antibody to ensure that equivalent levels of protein were loaded in each lane.

Viral RNA quantification and gene expression by real time RT-PCR

Total RNA was extracted using Tri reagent (Sigma, Saint Quentin Fallavier, France) according to the manufacturer's protocol. The RNA pellet was resuspended in 25 μl of RNase-free distilled water and stored at -80°C. The RNA was used for reverse transcription using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, Charbonnieres, France) according to the manufacturer's instructions. The reaction was carried out using 1 μg total RNA as template for the normalization of viral RNA to the amount of total RNA. The MaximaTM Probe/ROX qPCR Master Mix (2x) (Thermo Scientific) was used in qPCR experiment. Each reaction of 25 μL contained 400 nM of each primer, 200 nM of specific probe and 1x Maxima^{TM} Probe/ROX qPCR Master Mix. Primers and probe sequences are listed in S4 Table. The amplification conditions were 95 °C for 10 min followed by 45 amplification cycles of 95 °C for 15 s, 60 °C for 20 s and 72 °C for 30 s. The reactions were performed in an Applied Biosystem 7300 system. Real time data were analysed using the SDS software (Thermo Fischer Scientific). Viral RNA was quantified by comparing the sample's threshold

cycle (Ct) values with each virus RNA standard curve which was obtained as previously described [18, 19].

CHIKV replicon cell line based assay

Huh-7 cells stably transfected with the CHIKV-NCT (NonCytotoxic) replicon, were used to test the effects of SAMHD1 to CHIKV replication. CHIKV replicon cells were plated in six-well plates, treated with VLPs/Vpx and incubated at 37° C and 5% CO_2. After 24 and 48 h of incubation, Renilla Luciferase (*Rluc*) activity, expressed by the CHIKV replicon, was detected using the Renilla Luciferase assay (Promega, Charbonnière, France) according to the manufacturer's instructions. The luminescence signal, proportional to the CHIKV's RNA replication, was then measured using the *Modulus microplate luminometer* (Turner BioSystems, CA).

Plaque assay

Vero cells, grown to 70-80% confluence, were incubated with four separate, ten-fold, dilutions of viral supernatant and cultured at 37° C for 2 h. Then, a mix of nutriment solution with agar (Lonza) was added and the cells were maintained at 37° C for 6 days. For plaque counting, the cells were fixed with 3.7% formaldehyde and stained with 0.5% Crystal violet in 20% ethanol.

Data analysis and statistical methods

All data are presented as means ± standard deviation (SD). The Student's *t* test was used to determine the statistical significance. For all experiments, statistical significance was accepted at $p < 0.05$.

Results

Identification of differentially expressed proteins in infected HFF1 cells

To analyze changes in proteome response to viral infection, HFF1 cells were grown in either light or heavy labeled medium before being infected with CHIKV or ZIKV. The cells were harvested for nanoflow LC-MS analysis after 48 h post infection (hpi). Light and heavy mock-infected cells were used as a negative control. The proteomic analysis resulted in the identification of 2,132 and 2,696 differently expressed proteins in CHIKV- and ZIKV-infected cells, respectively (Tables S1 and S2). The expression of sixteen proteins was found to be significantly up-regulated in either CHIKV- or ZIKV-infected cells, with nine proteins that were expressed in both viruses (Table 1). Following a functional enrichment analysis, 17 annotations were found to be significantly enriched among up-regulated proteins in CHIKV-infected cells, whereas there were 11 significantly enriched annotations in ZIKV-infected cells (Table 2 and S3 Table). The enriched functional annotations for both CHIKV and ZIKV virus-infected cells included proteins involved in the IFN I signaling pathway, anti-viral defense responses to virus, negative regulation of the viral infection process and viral genome replication (Table 2 and S3 Table). Most of these proteins are encoded by interferon-stimulated genes (ISGs) and several represent IFN-induced proteins with tetratricopeptide repeats (IFITs), including IFIT1 (ISG56), and IFIT3 (ISG60). The functional relatedness of up-regulated these arbovirus-induced proteins is corroborated by their high interconnectivity in the STRING human functional interaction network (Fig 1). Interestingly, nine identical closely related proteins, MX1, IFIT1, IFIT3, ISG15, DDX58, STAT1, OAS3, EIF2AK2 and SAMHD1, were up-regulated in both infection models and, with the exception of the latter protein, all were interconnected in the functional network (Fig 1).

IFIT proteins affect CHIKV and ZIKV replication in HFF1 cells

As mentioned above, several IFITs were prominently up-regulated in arbovirus-infected HFF1 cells: IFIT1, 2 and 3 following CHIKV and IFIT1 and 3 following ZIKV infection, respectively (Table 1). These up-regulated proteins were selected for further investigation. Analysis of their expression levels by Western blotting analysis confirmed that the amounts of each of these proteins were increased in

both CHIKV and ZIKV-infected human fibroblasts in a time-dependent manner (Table 1 and Figs 2A and 2B). As expected, the expression level of IFIT2 in ZIKV infected cells was similar to that observed in mock-infected cells (Fig 2B), thus validating the results from the SILAC/MS analysis.

To study the functional role of IFITs proteins in the viral infection process, their genes were either overexpressed or their expression was suppressed in HFF1 cells using specific RNA silencing. The cells were subsequently infected with CHIKV or ZIKV after which the synthesis of viral RNA and the production of viral particles were analyzed. Viral RNA levels and virion production of both viruses were almost totally abrogated in infected fibroblasts that overexpressed either IFIT1 or IFIT3 (Figs 2C and 2D), while viral RNA synthesis and virion production were significantly increased both in IFIT1 and 3-silenced cells (Figs 2E and 2F). In keeping with our previous observations the modulation of the expression of IFIT2 only affects CHIKV replication (Figs 2C and 2E). These results indicate that certain IFITs family members play a significant role in the regulation of CHIKV and ZIKV replication.

SAMHD1 is up-regulated upon CHIKV and ZIKV infection via the production of type I interferons

The results from the SILAC/MS analysis showed significantly increased expression of SAMHD1 in HFF1 cells following both CHIKV and ZIKV infection. To validate these results, immunoblotting and RT-qPCR analysis were performed in infected cells. Western blotting analysis of CHIKV-, as well as ZIKV-infected cells showed that in both cases SAMHD1 expression was up-regulated in a time-dependent manner (Figs 3A and 3B) which was furthermore corroborated by data from RT-qPCR analysis (Figs 3C and 3D). Interestingly, Cribier *et al*. [20] reported that phosphorylation of SAMHD1 (pSAMHD1) is a key regulatory mechanism that inhibits the ability of SAMHD1 to block Human immunodeficiency virus type 1 (HIV-1) infection. We sought to verify whether the pSAMHD1 might also occur during the infection with an RNA virus. Immunoblotting analysis using an antibody specific for the pSAMHD1 revealed a clear induction of pSAMHD1 at 24 hpi. Its expression was also

detectable at 48 hpi albeit at reduced level (Figs 3A and 3B). These results suggest that SAMHD1 and/or its phosphorylated form exert their activities in both CHIKV and ZIKV infections.

It has been reported that type I IFN treatment up-regulates endogenous SAMHD1 expression in HEK293T cells, porcine macrophages and MARC-145 cells [26]. However, it is unclear whether SAMHD1 is also induced by IFNs in human skin fibroblasts. To address this question, HFF1 cells were treated by either type I (α and β) or type II (γ) IFN at different time points and SAMHD1 expression was detected by Western blotting analysis. This analysis revealed that only IFN type I, but not type II, treatment was able to induce SAMHD1 expression in these cells and similar to that observed following viral infection, the induction occurred in a time-dependent manner (Fig. 3E). Induction of SAMHD1 expression by both types of IFN followed different kinetics: upon induction with IFN-α, SAMHD1 expression reached maximal levels already after 24 h, which were observed only after 48 h of treatment of the cells with IFN-β.

HIV-2 Vpx protein induced SAMHD1 degradation inhibits CHIKV and ZIKV replication.

SAMHD1 was initially identified as a restriction factor that inhibits HIV-1 replication in resting cells. It has been shown that SAMHD1 is degraded by the accessory protein Vpx, ~~which is~~ encoded by simian immunodeficiency virus (SIV) and HIV-2, but not by HIV-1 itself [27, 28]. To determine the effect of Vpx on endogenous SAMHD1 levels in human skin fibroblasts, the cells were incubated with virus like particles (VLPs) that contain the Vpx protein of SIV [27], infected with CHIKV and expression levels of SAMHD1 were determined by Western blotting analysis. At 24 hpi SAMHD1 protein could no longer be detected in the VLP/Vpx treated cells (Fig 4A). Next, to assess whether Vpx-mediated SAMHD1 degradation affects replication of CHIKV and ZIKV, VLP/Vpx-treated or mock-treated HFF1 cells were infected with each of the viruses. At 48 hpi intracellular viral RNA levels, as well as titers of released virions, were severely reduced in VLP/Vpx-treated cells, infected with CHIKV or ZIKV (Figs 4B and 4C), pointing to an almost complete lack of viral RNA synthesis and virion production. The effect of VLP/Vpx treatment on the post-entry steps of CHIKV infection

cycle was confirmed using the Huh7-CHIKV replicon cell line that enables to specifically measure viral RNA replication. The results of this analysis show a highly significant decrease in *RLuc* activity, which is directly proportional to CHIKV RNA replication, with a stronger inhibition at 48 h post-treatment (Fig 4D). Together, these data demonstrate that degradation of SAMHD1 by SIV Vpx inhibits CHIKV and ZIKV infection and that it affects at least one of post entry step of the CHIKV infection cycle.

SAMHD1 enhances both CHIKV and ZIKV replication in HFF1 cells.

The data obtained thusfar indicate that SAMHD1 is an important pro-viral factor for both CHIKV and ZIKV. In contrast however, SAMHD1 has been reported to inhibit HIV-1 and Hepatitis B virus replication by depleting the intracellular pool of dNTPs to levels below those required to complete the reverse transcription step [29-33]. Thus, SAMHD1 seems to exert opposite effects on viruses using reverse transcription and arboviruses with a positive-strand RNA genome. To test this hypothesis, HFF1 cells stably expressing either an irrelevant control or a SAMHD1 mRNA were generated using transduction with a lentiviral vector. Based on the expression of the EGFP marker that had been inserted in the constructs, results from flowcytometry experiments showed that control and SAMHD1 RNA were expressed by almost all transduced cells (Fig 5A). SAMHD1 over-expression was also confirmed by Western blotting analysis. Next, parental HFF1 cells and both obtained cell lines were either mock infected or infected with CHIKV or ZIKV. The presence of the SAMHD1 expressing transgene resulted in a significant and prominent increase, close to 2 logs, both in intracellular viral RNA copy numbers and titers of CHIKV and ZIKV virions (Figs 5B and 5C). The impact of SAMHD1 over-expression on viral replication was further examined using U937 cells, a cell line that is not permissive for CHIKV [34] and its variant that stably overexpresses SAMHD1. The results were obtained with both viruses were similar in that wild type U937 cells were poorly infected with CHIKV (Fig. 5D) or ZIKV (Fig. 5E). Yet for both viruses increased viral RNA levels and viral titers were observed in the SAMHD1-expressing cells (Figs 5D and 5E). Finally, these results were corroborated by siRNA-mediated silencing of SAMHD1 in HFF1 cells. Intracellular viral RNA levels and titers of both viruses were strongly decreased upon SAMHD1 silencing. The observed inhibition in SAMHD1-

silenced cells reached close to 99 and 95% for CHIKV and ZIKV, respectively. In contrast, the inhibition in control-siRNA treated cells was negligible (Figs 5F and 5G). Together, these results strongly suggest that SAMHD1 increases the replication of positive-strand RNA viruses such as CHIKV and ZIKV.

Discussion

Arboviruses, like CHIKV and ZIKV, (belonging to the alphavirus and flavivirus family, respectively,) are transmitted by the genus *Aedes* to human via a bite in the skin during bloodfeeding. The ensuing viral entry, RNA replication and release of viral particles is the result of complex interactions between these viruses and their human host cells. The identification of host proteins that are differentially expressed during an infection with CHIKV and ZIKV will provide a better understanding of the processes leading to the pathology caused by these viruses and may open approaches leading to therapeutic intervention. To that aim, we have determined the proteomic profiles of CHIKV and ZIKV-infected human skin fibroblasts, a main site of entry for arboviruses following the bite of an infected mosquito [18, 19]. The proteomics analysis of both arbovirus-infected HFF1 cells revealed 2,132 and 2,696 identified proteins in CHIKV and ZIKV-infected cells, respectively. From these sixteen proteins, nine of them were common for both of viruses, were significantly over-expressed, as compared to the non-infected control cells and most were encoded by ISGs.

Both alphaviruses and flaviruses trigger the IFN type I signaling pathway, leading to the transcription of ISGs, whose protein products prevent or suppress the infection of these pathogens [35]. Accordingly, in the present study CHIKV was found to induce the expression of the IFIT1, 2 and 3 proteins. IFITs are ISGs that can inhibit viral replication through multiple mechanisms, including suppression of translation initiation [36], binding of uncapped or incompletely capped viral RNA [37, 38], as well as sequestration of viral proteins or RNAs in the cytoplasm of the host cell [39, 40]. Rabbani and colleagues have shown that IFITs have specific antiviral functions in response to human parainfluenza virus type 3 infection [41] and, similarly, Reynaud *et al*. reported that IFIT1 interferes

with the translation and replication of several alphaviruses [42]. Our results confirm and extend these observations by showing that the forced expression of IFIT1, IFIT2 or IFIT3 in human fibroblasts completely inhibited the replication of CHIKV and that individual knock-down of any of these genes facilitated virus infection. Similar data were obtained with respect to the inhibition of ZIKV replication except that IFIT2, does not seem to be involved in this process, at least not in the human skin fibroblast model used here.

SAMHD1 is a deoxynucleoside triphosphate triphosphohydrolase protein with a sterile α-motif (SAM) and an HD domain that harbors the active site of the protein [43]. It was recently identified as a HIV-1 restriction factor in resting cells and has been shown to be degraded by the accessory factor Vpx through a proteasome-dependent mechanism [29, 44, 45]. Several recent studies have investigated the role of the SAMHD1 protein in inhibiting virus infectivity. It has been proposed that the inhibitory effect of SAMHD1 on HIV-1 replication is due to by its dNTPase activity [46]; and alternatively that SAMHD1, or an associated protein could restrict HIV-1 through an RNase activity [47, 48]. These findings notwithstanding, no information is as yet available regarding the role of SAMHD1 during arbovirus infection. In contrast to the results from the above-mentioned studies that have underscored the role of SAMHD1 as a restriction factor able to inhibit the replication of reverse transcription-required viruses [32, 33, 49-52], the present study is the first to demonstrate that SAMHD1 has proviral role in CHIKV and ZIKV infection. First, Vpx/VLP-mediated SAMHD1 degradation was found to cause a prominent decrease in the replication of and virion production by both viruses. Secondly, the same treatment reduces replication of CHIKV replicon RNA. Finally, SAMHD1 over-expression facilitates CHIKV and ZIKV replication in several cell lines. The inconsistency between this finding and the clear evidence about anti-viral functions of SAMHD1 is most likely due to differences in the replication process used by these viruses: CHIKV and ZIKV, unlike HIV-1 and HBV, do not require the reverse transcription step for their replication.

In conclusion, our work highlights the crucial role of IFITs family members in the regulation of CHIKV and ZIKV replication. Our data also provide novel evidence on the role of SAMHD1 in arbovirus infection of human skin cells. However, the precise molecular mechanisms by which

SAMHD1 increases CHIKV and ZIKV replication remains to be investigated. The modulation of SAMHD1 expression found in our study might be a strategy of CHIKV and ZIKV to facilitate their replication. This study opens therefore new exciting perspectives for the study of the precise role of SAMHD1 in arbovirus infection.

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Supporting information captions

S1 Table. SILAC analysis raw data of CHIKV-infected cells

S2 Table. SILAC analysis raw data of ZIKV-infected cells

S3 Table. Functional annotation of CHIKV- and ZIKV-infected cells

S4 Table. Primers and probes for viral detection used in this study.

Table 1. Proteins significantly up-regulated in CHIKV- and ZIKV-infected cells.

Proteoform name	Gene name	UniprotID	Average log2 ratio
Chikungunya virus infected cells proteins			
Interferon-induced GTP-binding protein Mx1	MX1	P20591	5.043
Interferon-induced protein with tetratricopeptide repeats 3	IFIT3	O14879	3.591
Interferon-induced protein with tetratricopeptide repeats 1	IFIT1	P09914	3.449
Interferon-induced protein with tetratricopeptide repeats 2	IFIT2	P09913	2.954
Ubiquitin-like protein ISG15	ISG15	P05161	2.580
Probable ATP-dependent RNA helicase DDX58	DDX58	O95786	2.444
Signal transducer and activator of transcription 1-alpha/beta	STAT1	P42224	1.659
2'-5'-oligoadenylate synthase 3	OAS3	Q9Y6K5	1.626
Deoxynucleoside triphosphate triphosphohydrolase SAMHD1	SAMHD1	Q9Y3Z3	1.208
HLA class I histocompatibility antigen, B-45 alpha chain	HLA-B	P30483	1.119
Cytosol aminopeptidase	LAP3	P28838	1.049
Interferon-induced 35 kDa protein	IFI35	P80217	1.015
E3 ubiquitin-protein ligase RNF213	RNF213	Q63HN8	0.895
Gamma-interferon-inducible protein 16	IFI16	Q16666	0.878
Double-stranded RNA-activated protein kinase	EIF2AK2	P19525	0.838
HLA class I histocompatibility antigen, A-69 alpha chain	HLA-A	P10316	0.752
Zika virus infected cells proteins			
Interferon-induced GTP-binding protein Mx1	MX1	P20591	5.622
Interferon-induced protein with tetratricopeptide repeats 1	IFIT1	P09914	3.362
Interferon-induced protein with tetratricopeptide repeats 3	IFIT3	O14879	3.076
Ubiquitin-like protein ISG15	ISG15	P05161	2.430
2-5-oligoadenylate synthase 3	OAS3	Q9Y6K5	2.190
Poly [ADP-ribose] polymerase 9	PARP9	Q8IXQ6-2	2.118
Phospholipid scramblase 1	PLSCR1	O15162	2.085
Probable ATP-dependent RNA helicase DDX58	DDX58	O95786	1.974
Deoxynucleoside triphosphate triphosphohydrolase SAMHD1	SAMHD1	Q9Y3Z3	1.739

Signal transducer and activator of transcription 1-alpha/beta	STAT1	P42224	1.440
Synaptopodin-2	SYNPO2	Q9UMS6	1.415
E3 ubiquitin-protein ligase DTX3L	DTX3L	Q8TDB6	1.397
2-5-oligoadenylate synthase 1	OAS1	P00973	1.135
E3 ubiquitin-protein ligase TRIM21	TRIM21	P19474	1.085
Double-stranded RNA-activated protein kinase	EIF2AK2	P19525	1.071
Interferon-induced GTP-binding protein Mx2	MX2	P20592	0.993

Table 2. Functional annotations: Significant pathways identified in CHIKV- and ZIKV-infected cells.

Chikungunya Virus

Biological processes

GO ID	GO term	Corrected P-value
GO:0060337	Type I interferon signaling pathway	9.94E-21
GO:0051607	Defense response to virus	3.53E-16
GO:0048525	Negative regulation of virus infection process	1.37E-10
GO:0045071	Negative regulation of viral genome replication	4.25E-10
GO:0031348	Negative regulation of defense response	0.0000326
GO:0035455	Response to interferon-alpha	0.000153
GO:0045088	Regulation of innate immune response	0.000212
GO:0060333	Interferon-gamma-mediated signaling pathway	0.000249
GO:0002698	Negative regulation of immune effector process	0.000407
GO:0043330	Response to exogenous dsRNA	0.00281
GO:0050688	Regulation of defense response to virus	0.00602

KEGG

Pathway ID	Pathway name	Corrected P-value
KEGG:05168	Herpes simplex infection	4.12E-08
KEGG:05160	Hepatitis C	0.0000275
KEGG:05162	Measles	0.0000286
KEGG:05164	Influenza A	0.000101
KEGG:05169	Epstein-Barr virus infection	0.00553

Reactome

Pathway ID	Pathway name	Corrected P-value
REAC:913531	Interferon Signaling	5.41E-19

Zika Virus

Biological processes

GO ID	GO term	Corrected P-value
GO:0051607	Defense response to virus	5.74E-16
GO:0071357	Cellular response to type I interferon	2.77E-15
GO:0048525	Negative regulation of viral process	3.85E-15
GO:1903901	Negative regulation of viral life cycle	7.82E-13
GO:0035455	Response to interferon-alpha	0.000000633
GO:0034341	Response to interferon-gamma	0.0000822
GO:0043330	Response to exogenous dsRNA	0.00456
GO:0045088	Regulation of innate immune response	0.00899

KEGG

Pathway ID	Pathway name	Corrected P-value
KEGG:05160	Hepatitis C	0.0104
KEGG:05162	Measles	0.0108

Reactome

Pathway ID	Pathway name	Corrected P-value
REAC:913531	Interferon Signaling	1.22E-15

Figure 1

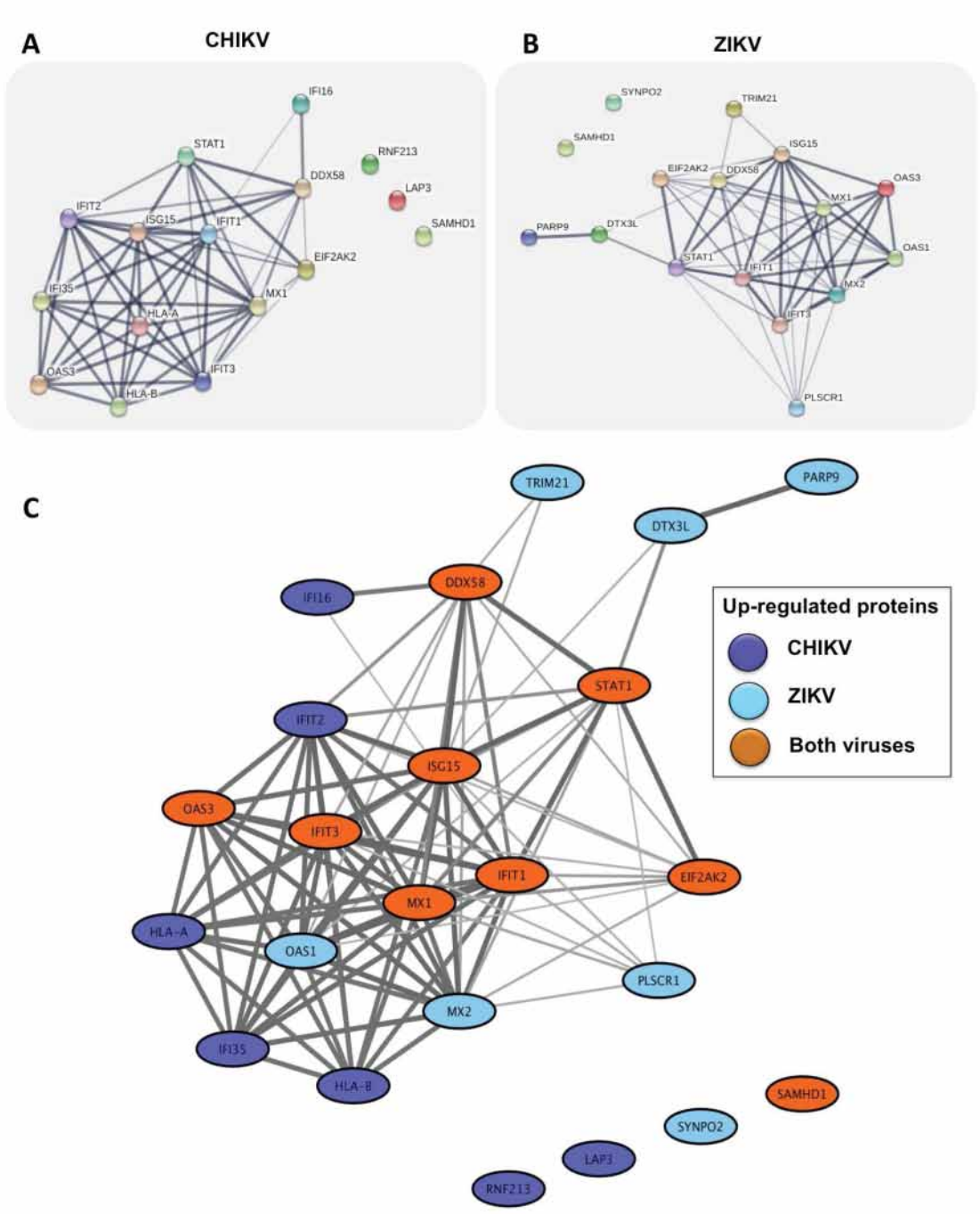
A
CHIKV
IFI16
STAT1
RNF213
DDX58
LAP3
IFIT2
ISG15
IFIT1
SAMHD1
EIF2AK2
IFI35
MX1
HLA-A
OAS3
IFIT3
HLA-B
B
ZIKV
SYNPO2
TRIM21
SAMHD1
ISG15
EIF2AK2
DDX58
OAS3
MX1
PARP9
DTX3L
OAS1
STAT1
IFIT1
MX2
IFIT3
PLSCR1
C
TRIM21
PARP9
DTX3L
DDX58
IFI16
STAT1
IFIT2
ISG15
OAS3
IFIT3
IFIT1
EIF2AK2
MX1
HLA-A
OAS1
PLSCR1
MX2
IFI35
HLA-B
SAMHD1
SYNPO2
LAP3
RNF213
Up-regulated proteins
CHIKV
ZIKV
Both viruses

Figure 1. Network representation of proteins significantly up-regulated in CHIKV and ZIKV infected HFF1 cells.

(A) Functional interaction network among significantly up-regulated proteins in CHIKV-infected cells. (B) Functional interaction network among significantly up-regulated proteins in ZIKV-infected cells. (C) Merged functional interaction network among all significantly up-regulated proteins in both CHIKV and ZIKV infected cells. Proteins are represented as nodes and functional relationships by edges. The thickness of edges is proportional to the confidence level of the functional relationship.

Figure 2

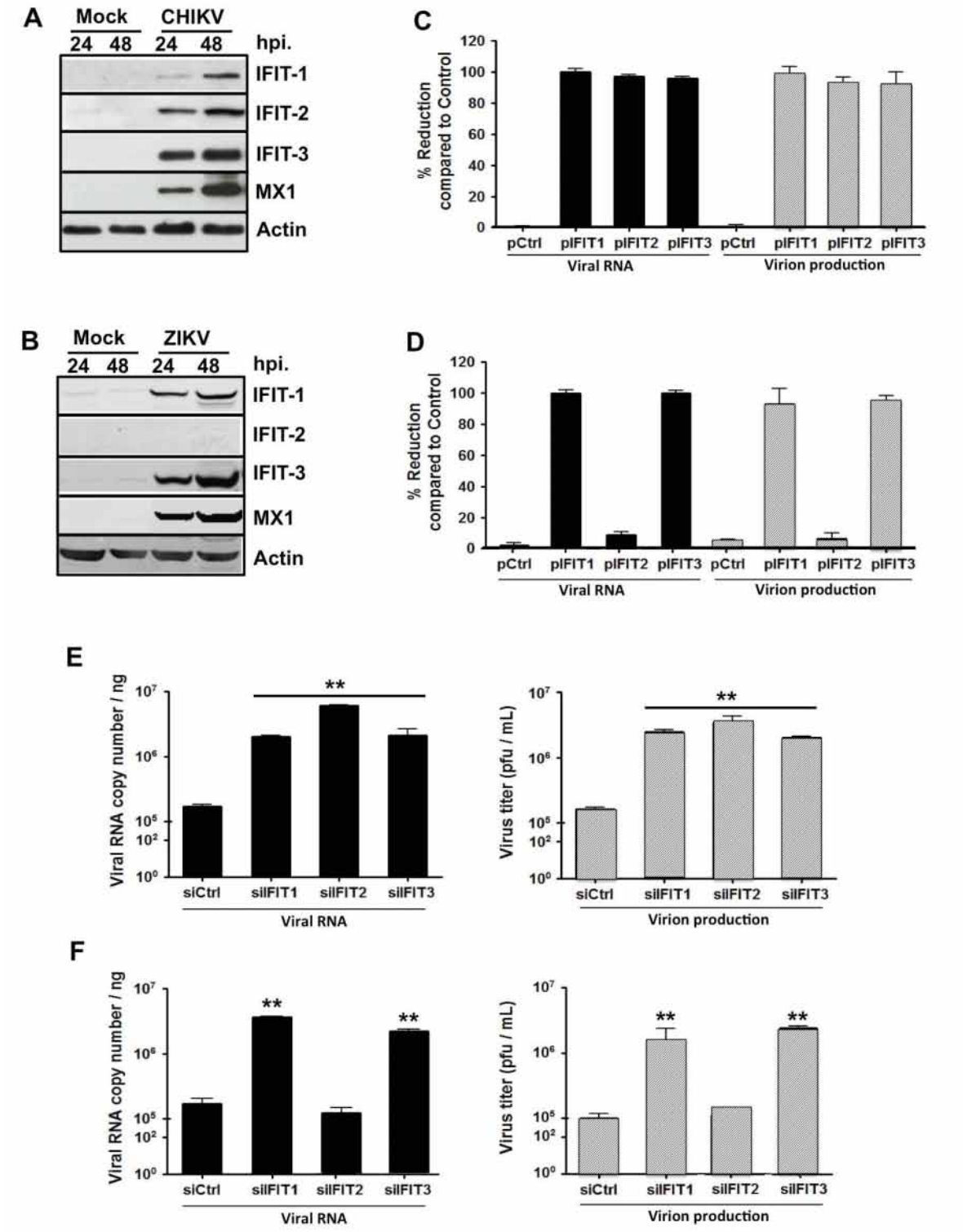
A
Mock
CHIKV
24 48 24 48
hpi.
IFIT-1
IFIT-2
IFIT-3
MX1
Actin
B
Mock
ZIKV
24 48 24 48
hpi.
IFIT-1
IFIT-2
IFIT-3
MX1
Actin
C
% Reduction compared to Control
120
100
80
60
40
20
0
pCtrl
pIFIT1
pIFIT2
pIFIT3
pCtrl
pIFIT1
pIFIT2
pIFIT3
Viral RNA
Virion production
D
% Reduction compared to Control
120
100
80
60
40
20
0
pCtrl
pIFIT1
pIFIT2
pIFIT3
pCtrl
pIFIT1
pIFIT2
pIFIT3
Viral RNA
Virion production
E
Viral RNA copy number / ng
10^7
10^6
10^5
10^2
10^0
**
siCtrl
siIFIT1
siIFIT2
siIFIT3
Viral RNA
Virus titer (pfu / mL)
10^7
10^6
10^5
10^2
10^0
**
siCtrl
siIFIT1
siIFIT2
siIFIT3
Virion production
F
Viral RNA copy number / ng
10^7
10^6
10^5
10^2
10^0
**
**
siCtrl
siIFIT1
siIFIT2
siIFIT3
Viral RNA
Virus titer (pfu / mL)
10^7
10^6
10^5
10^2
10^0
**
**
siCtrl
siIFIT1
siIFIT2
siIFIT3
Virion production

Figure 2. IFITs affect CHIKV and ZIKV infection in HFF1 cells.

(A) HFF1 cells were infected with CHIKV at an MOI 1; at 24 and 48 hpi infected cells were lysed with TETN-150 and analyzed by immunoblotting against IFIT1, IFIT2, IFIT3, MX1 and β-actin. (B) The experiment was similar to CHIKV but using ZIKV at an MOI 1. For both panels A and B mock-infected cells were used as control (C) CHIKV and (D) ZIKV virus RNA and infectious virus production were determined at 48 hpi in HFF1 cells transfected with plasmid expressing either control RNA (pCtrl) or mRNA encoding for IFIT1 (pIFIT1), pIFIT2 and pIFIT3 by real time RT-PCR (black bar) and plaque assay (gray bar), respectively. The percentage of reduction at the presence of each plasmid was calculated using formula [1-(R/C)]*100 where C and R designate experimental values (RNA copy numbers or plaque numbers) at the presence of pCtrl and pIFIT, respectively. (E) CHIKV and (F) ZIKV virus RNA and infectious virus production were determined at 48 hpi in cells transfected with control siRNA (siCtrl) or siRNA specific for IFIT1 (siIFIT1), siIFIT2 and siIFIT3 by real time RT-PCR (black bar) and plaque assay (gray bar), respectively. The data represent mean ± SD from three independent experiments. **, $p < 0.01$ when compared to siCtrl transfected cells.

Figure 3

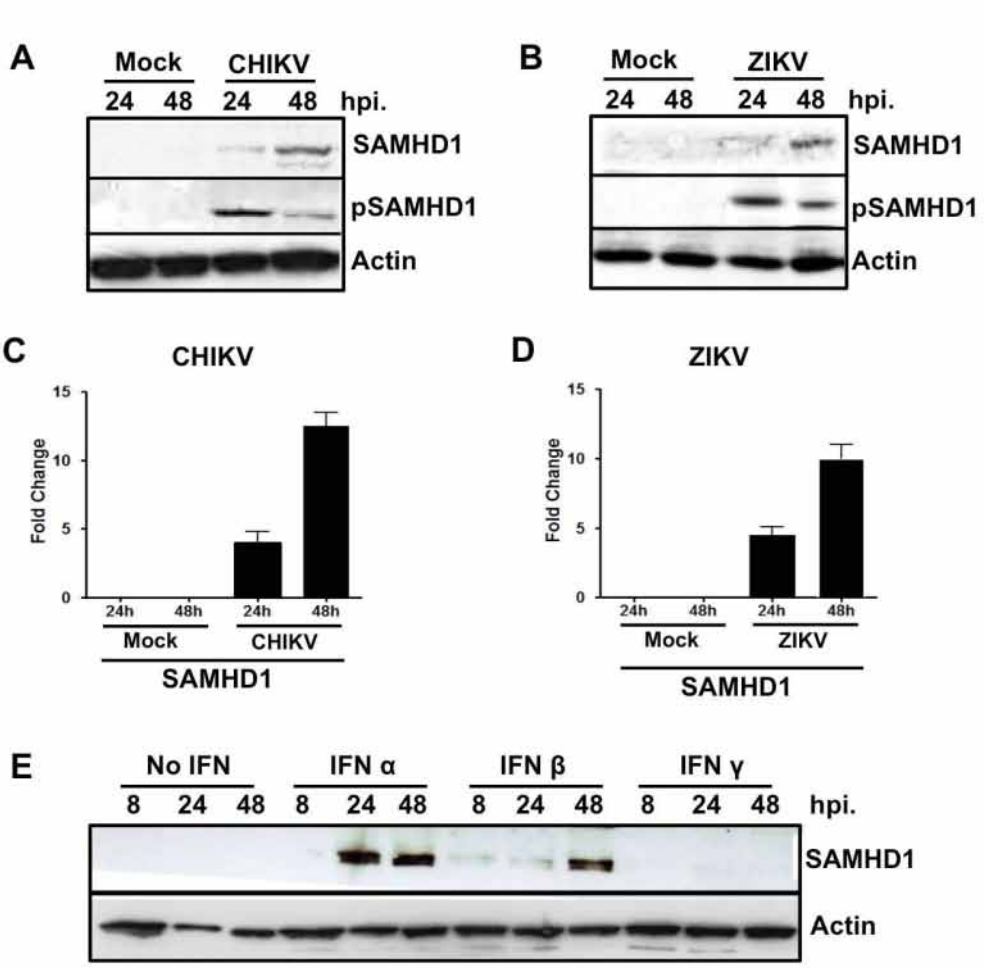
A
Mock
CHIKV
24 48 24 48 hpi.
SAMHD1
pSAMHD1
Actin
B
Mock
ZIKV
24 48 24 48 hpi.
SAMHD1
pSAMHD1
Actin
C
CHIKV
Fold Change
15
10
5
0
24h 48h 24h 48h
Mock
CHIKV
SAMHD1
D
ZIKV
Fold Change
15
10
5
0
24h 48h 24h 48h
Mock
ZIKV
SAMHD1
E
No IFN
IFN α
IFN β
IFN γ
8 24 48 8 24 48 8 24 48 8 24 48 hpi.
SAMHD1
Actin

Figure 3. SAMHD1 is up-regulated upon CHIKV and ZIKV infection and by treatment with type I interferons.

HFF1 cells infected with CHIKV or ZIKV at an MOI 1; control cells were mock-infected. At 24 or 48 hpi cells were lysed with TETN-150 and analyzed by immunoblotting using antibodies against SAMHD1, phospho-specific Thr592 SAMHD1 and β-actin (A and B). SAMHD1 mRNA levels of the same samples were analyzed by real time PCR (C and D). (E) HFF1 cells were treated by 1000 U/ml of IFN α, -β or –γ. At 8, 24 or 48 h post treatment cells were lysed with TETN-150 and analyzed by immunoblotting using antibodies against SAMHD1 and β-actin.

Figure 4

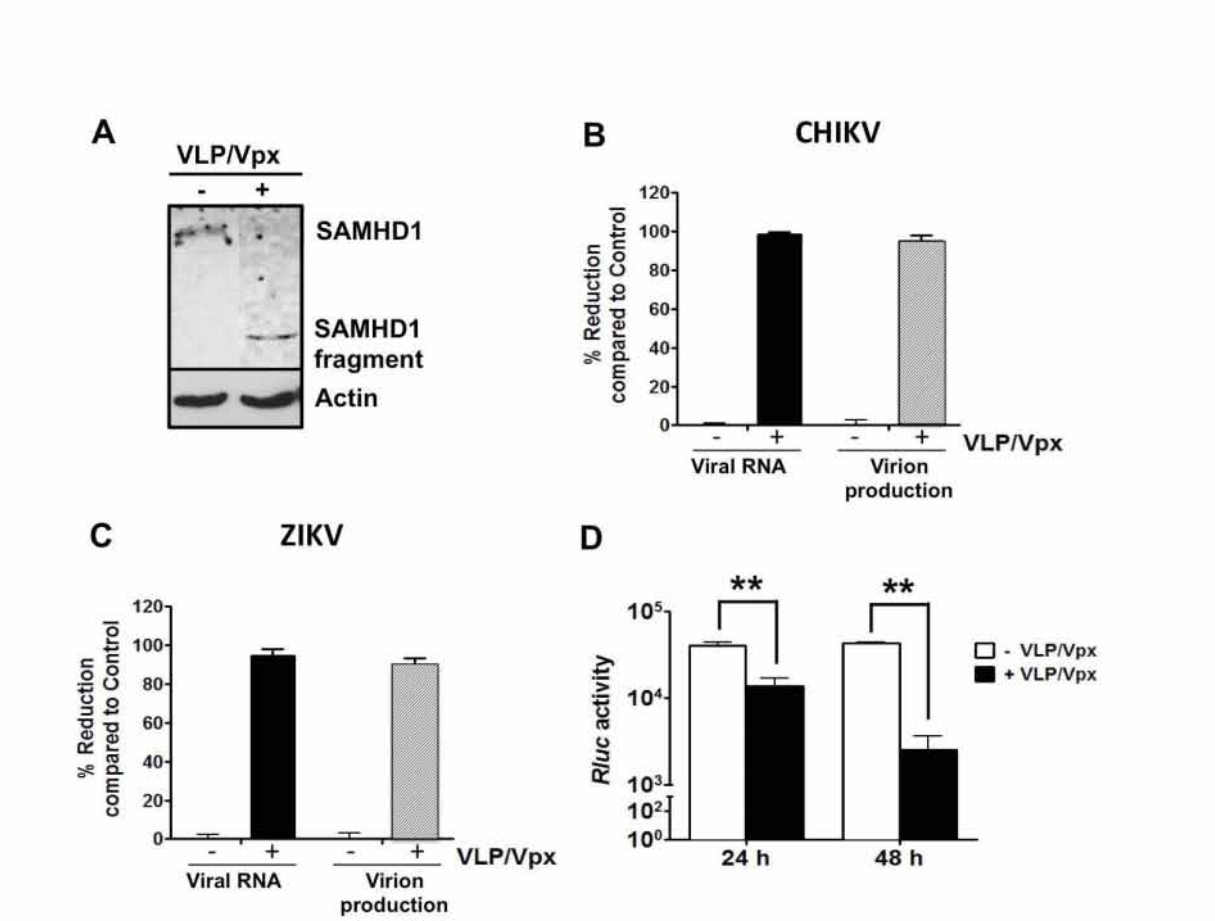
A
VLP/Vpx
-
+
SAMHD1
SAMHD1 fragment
Actin
B
CHIKV
% Reduction compared to Control
120
100
80
60
40
20
0
-
+
-
+
VLP/Vpx
Viral RNA
Virion production
C
ZIKV
% Reduction compared to Control
120
100
80
60
40
20
0
-
+
-
+
VLP/Vpx
Viral RNA
Virion production
D
**
**
Rluc activity
10^5
10^4
10^3
10^2
10^0
- VLP/Vpx
+ VLP/Vpx
24 h
48 h

Figure 4. Vpx induced SAMHD1 degradation decreases CHIKV and ZIKV replication.

(A) Mock (-) or VLP/Vpx-treated (+) HFF1 cells were infected with CHIKV for 24 h. Infected cells were lysed with TETN-150 and analyzed by immunoblotting using antibodies against SAMHD1 and and β-actin. (B and C) Vpx treated and mock-treated HFF1 cells were infected with CHIKV or ZIKV at MOI 1. After 48 h, intracellular virus RNA levels and infectious virus production were measured by real time RT-PCR (black bar) and plaque assay (gray bar), respectively. The percentage of reduction at the presence of Vpx was calculated using formula [1-(R/C)]*100 where C and R designate experimental values (RNA copy numbers or plaque numbers) at the presence of control and Vpx, respectively. (D) Stable Huh-7 cell line harboring CHIKV-NCT replicon was untreated or treated with Vpx. 24 and 48 h post treatment cells were lyzed and Renilla Luciferase (*Rluc*) activity was measured. Relative *Rluc* activity expressed by the CHIKV replicon represents CHIKV's RNA replication. Values are normalized according to protein contents of the cell extract and correspond to the mean of triplicate ± SD. The data represent mean ± SD from three independent experiments. **, p-value < 0.01 when compared to untreated cells.

Figure 5

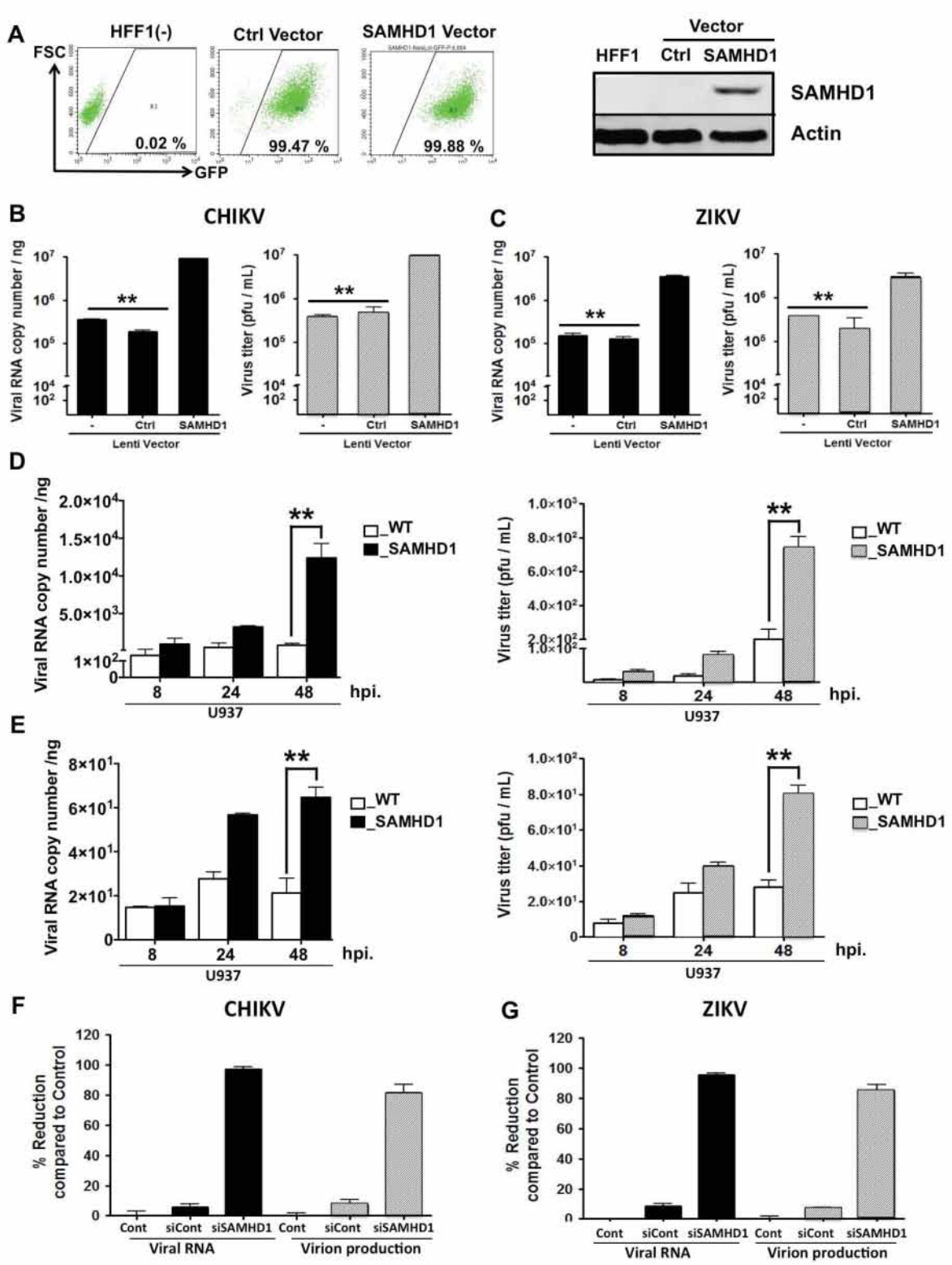
A
HFF1(-)
Ctrl Vector
SAMHD1 Vector
FSC
GFP
0.02 %
99.47 %
99.88 %
Vector
HFF1
Ctrl
SAMHD1
SAMHD1
Actin
B
CHIKV
C
ZIKV
Viral RNA copy number / ng
Virus titer (pfu / mL)
Lenti Vector
D
Viral RNA copy number /ng
Virus titer (pfu / mL)
_WT
_SAMHD1
hpi.
U937
E
F
CHIKV
G
ZIKV
% Reduction compared to Control
Cont
siCont
siSAMHD1
Viral RNA
Virion production

Figure 5. SAMHD1 over-expression enhances CHIKV and ZIKV replication

(A) HFF1 were transduced by either control (Ctrl)- or SAMHD1- GFP lentiviral vector to generate cell lines stably expressing EGFP and control or SAMHD1 mRNAs. Transduced cells were sort by FACSAria III and obtained cell lines were validated for the EFGP expression by flow cytometry and for SAMHD1 expression by Western blotting analysis. (B and C) Normal (-), stably expressing control (Ctrl) or SAMHD1 mRNA HFF1 cells were infected with CHIKV or ZIKV at MOI 1. At 48 hpi intracellular virus RNA was quantified by real time RT-PCR (black bar); virus titers were measured using plaque assay (gray bar). (D and E) U937 WT or stably SAMHD1 overexpressed cells were infected with CHIKV or ZIKV at MOI 1. At 8, 24 and 48 hpi the intracellular virus RNAs were quantified by RT-PCR (black bar); virus titers were measured using plaque assay (gray bar). (F and G) Amounts of CHIKV and ZIKV RNAs and infectious virus production were analyzed at 24 hpi. in HFF1 cells that were mock-transfected (Cont) ortransfected either with control siRNA (siCtrl) or siRNA specific for SAMHD1 (siSAMHD1). Virus RNAs were quantified using real time RT-PCR (black bar) and titers using plaque assay (gray bar). Reduction at the presence of siCont and siSAMHD1 were calculated using formula [1-(R/C)]*100 where C and R designate experimental values (RNA copy numbers or plaque numbers) at the presence of cell untreated control (C) and siCont or siSAMHD1 treated cells (R), respectively. The data represent mean ± SD from three independent experiments. **, p-value < 0.01 when compared to control cells.

CHAPTER IV:

Aedes Aegypti SALIVA ENHANCES CHIKUNGUNYA VIRUS REPLICATION IN HUMAN SKIN FIBROBLASTS

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Aedes Aegypti Saliva Enhances Chikungunya Virus Replication in Human Skin Fibroblasts

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Key Words:	Chikungunya, skin fibroblast, Saliva, innate immunity, Aedes aegypti

Aedes Aegypti Saliva Enhances Chikungunya Virus Replication in Human Skin Fibroblasts

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Abbreviations: *Ae., Aedes; CHIKV, chikungunya virus; IFI, interferon-inducible; IFNs, interferons; SGE, salivary gland extract*

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TO THE EDITOR

Chikungunya virus (CHIKV), a mosquito-borne alphavirus, has caused large outbreaks of disease throughout Asia, Africa and several islands in the Indian Ocean with recent epidemics in the Americas (Gallian *et al.*, 2017). CHIKV is the agent of an acute febrile illness characterized by myalgia, rash, severe joint pain and, often debilitating, complications that can persist for years (Oviedo-Pastrana *et al.*, 2017). Transmission of CHIKV occurs through the bite of an infected *Aedes (Ae.)* mosquito when the virus is injected, together with mosquito saliva, into the skin of the human host. The mosquito salivary glands secrete various pharmacologically active molecules that contribute to successful blood feeding by inhibiting host hemostasis, inflammation and immune responses (Wichit et al., 2016). In addition, the presence of saliva during CHIKV infection was found to significantly suppress the expression of various inflammatory genes and the production of chemokines (Agarwal et al., 2016).

Human dermal fibroblasts have been reported to be susceptible to CHIKV replication *in vitro* (Sourisseau et al., 2007) and we have recently demonstrated that these cells contained the highest concentration of CHIKV antigens following viral infection *via* the skin (Ekchariyawat et al., 2015). In the present study, we have determined the effect of *Ae. aegypti* saliva on CHIKV replication in human skin fibroblasts which mimics the events that occur during natural transmission. To this end, the latter cells were infected with CHIKV in the absence or presence of 1 μg/ml of *Ae. aegypti* saliva which was maintained throughout the infection. At 48 hours post infection a significant increase of more than 2 logs in the expression of viral transcripts, measured by real-time quantitative PCR, was observed in fibroblasts infected with CHIKV in the presence of the saliva, as compared with that in cells infected with virus alone (Figure 1a). Moreover, results from a plaque assay confirmed the strong induction in the release of infectious CHIKV particles which increased in a time-dependent manner (Figure 1b). These data support previous observations showing that *Ae. aegypti* saliva has an

important role in the inhibition of an antiviral immune response by the host by creating an environment that favors the replication of CHIKV (Agarwal et al., 2016).

Type I interferons (IFNs) are known to combat viruses during viral infections and it has been well-established that CHIKV infection elicits a type I IFN response alongside the production of other pro-inflammatory cytokines (Lum and Ng, 2015). In the present study, CHIKV-infected human fibroblasts were found to express significantly increased levels of many type I IFN-responsive genes, as demonstrated by specific PCR array analysis. In contrast however, these genes were markedly decreased in cells infected with CHIKV in the presence of mosquito saliva (Figure 2a). The expression of the interferon-inducible (IFI) genes IFI6, IFI16, IFI27, IFI30 and IFIH1 (MDA5), as well as STAT 2, TNFSF 10 (TRAIL) and PRKRA, were further validated by real-time quantitative PCR (Figure 2b). As expected, the differential expression levels of the genes of interest strongly correlated with the microarray data, confirming that their expression levels were induced following challenge of the cells with the virus only and down-regulated in the presence of saliva. In contrast, and used as a negative control, expression levels of the PRKRA did not change under either experimental condition in accordance with the microarray data.

Following CHIKV infection, viral RNA is recognized by cytosolic RNA sensors that trigger the expression of various pro-inflammatory genes, resulting in an anti-viral type I IFN response. In particular, IFIH1 efficiently detects dsRNAs which leads to activation of the IRF3-dependent signaling pathways and, ultimately, type I IFN production. Consequently, triggering of the canonical pathway of IFN type I signalling through JAK-STAT activation results in the assembly of the ISGF3 complex that translocates to the nucleus to activate gene transcription (Schwartz and Albert, 2010). These concerted activation pathways converge to restrict CHIKV replication. Support for this notion is provided by the recent observation that alphavirus-infected cells were unable to induce a type I IFN response in the absence of

MDA5 (Akhrymuk et al., 2016). Moreover, the enhanced TNFSF 10 expression in CHIKV-infected fibroblasts is reminiscent of published results showing that triggering of the STAT1 signaling cascade by HIV-1 Vpr also enhanced expression of this molecule, which would presumably help to eliminate HIV-1-infected cells through TRAIL-mediated cell death (Zahoor et al., 2014). Therefore, the decrease in the expression of these molecules by mosquito saliva is meant to impair the host immune responses which, as a consequence, is advantageous to increased viral replication.

As a typical adaptive immune response does not develop immediately following infection, the innate immune system seems to be capable of controlling CHIKV infection. Concordantly, in this study, most of down-regulated genes after skin cell challenged with saliva belong to those of innate immune response. IFI6, IFI16, IFI27 and IFI30 were significantly down-regulated after challenged with saliva. As shown previously, increased IFI6 expression levels are associated with a strong decrease in yellow fever, Dengue type 2, as well as West Nile, virus infection and IFI6 was found to also limit HCV entry and replication in human hepatoma cells (Meyer et al., 2015). It is of note that IFI6 and IFI27 are two related proteins belonging to the FAM14 family on the basis of sequence similarity and that are commonly and concomitantly induced by IFNs. However, unlike IFI6, the latter molecule is not known to be involved in the modulation of virus infection. Another IFI gene induced in CHIKV infected skin cells, IFI16, reportedly acts as a restriction factor for several other DNA viruses (Orzalli et al., 2015), whereas it also induces anti-viral inflammasome activity against KSHV and EBV (Roy et al., 2016).

To our knowledge, this is the first study showing the importance of *Ae. aegypti* saliva in promoting CHIKV infection via the reduction of genes involved in type I IFN secretion by infected human skin fibroblasts although the elucidation of the molecular mechanism underlying this process needs further investigation.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

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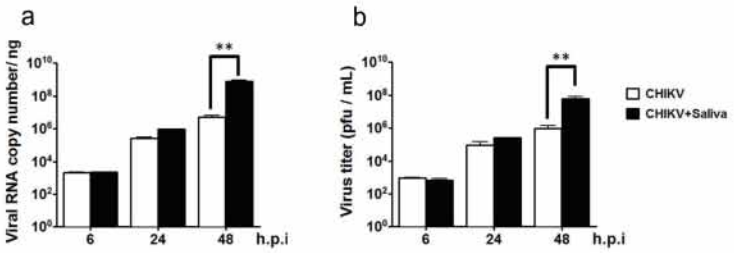
FIGURE LEGENDS

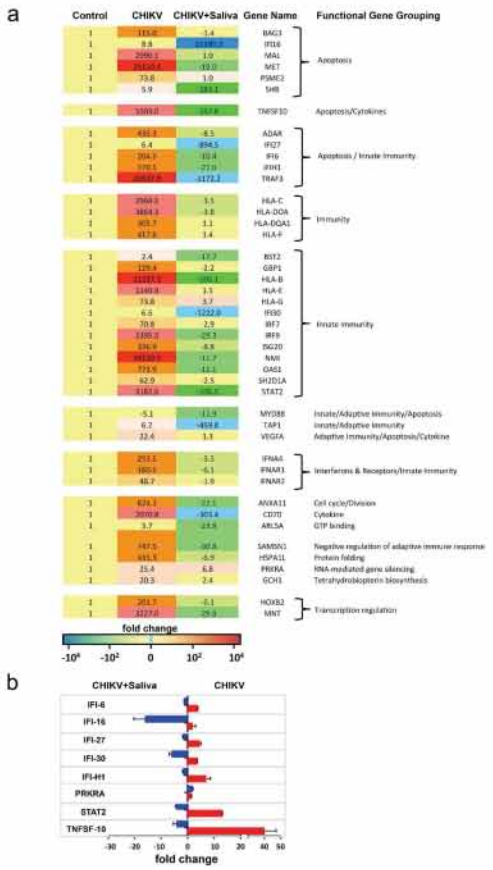
Figure 1. *Ae. aegypti* saliva enhances CHIKV replication in human skin fibroblasts. Cells were infected with CHIKV at a multiplicity of infection of 1 in the presence or absence of 1 µg/ml *Ae. aegypti* saliva. After 6, 24 and 48 hours, intracellular viral RNA and infectious virus production were quantified by (**a**) real-time quantitative RT-PCR and (**b**) plaque assay, respectively. Data are representative of three independent experiments, each performed in duplicate, ± standard deviation of the mean. Wilcoxon-Mann-Whitney test was used to analyze the difference between sets of data. A value of $p < 0.01$ was considered significant. ** p-values < 0.01.

Figure 2. Expression of genes involved in human type I interferon response regulation by CHIKV in human skin fibroblasts. Cells were exposed to CHIKV at a multiplicity of infection of 1 in the presence or absence of 1 µg/ml *Ae. aegypti* saliva. (**a**) The expression of significantly up- or down-regulated genes involved in the human type I interferon response was measured by PCR array. (**b**) mRNA levels of the indicated gene were validated by real-time quantitative RT-PCR. Results are expressed as fold change of transcripts in CHIKV-infected human skin fibroblasts in the presence or absence of saliva, relative to those in mock-infected cells (Control).



Ae. aegypti saliva enhances CHIKV replication in human skin fibroblasts. Cells were infected with CHIKV at a multiplicity of infection of 1 in the presence or absence of 1 µg/ml *Ae. aegypti* saliva. After 6, 24 and 48 hours, intracellular viral RNA and infectious virus production were quantified by (a) real-time quantitative RT-PCR and (b) plaque assay, respectively. Data are representative of three independent experiments, each performed in duplicate, ± standard deviation of the mean. Wilcoxon-Mann-Whitney test was used to analyze the difference between sets of data. A value of $p < 0.01$ was considered significant. ** p-values < 0.01.

355x139mm (300 x 300 DPI)



Expression of genes involved in human type I interferon response regulation by CHIKV in human skin fibroblasts. Cells were exposed to CHIKV at a multiplicity of infection of 1 in the presence or absence of 1 µg/ml *Ae. aegypti* saliva. (a) The expression of significantly up- or down-regulated genes involved in the human type I interferon response was measured by PCR array. (b) mRNA levels of the indicated gene were validated by real-time quantitative RT-PCR. Results are expressed as fold change of transcripts in CHIKV-infected human skin fibroblasts in the presence or absence of saliva, relative to those in mock-infected cells (Control).

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CHAPTER V:
CONCLUSIONS AND PERSPECTIVES

Despite the global distribution of arboviruses, especially Chikungunya virus (CHIKV), the management of patients affected by disease caused by the virus is still performed during an exclusively symptomatic period because of the absence of a specific antiviral treatment and licensed vaccine. The re-emergence of CHIKV in 2005 caused millions cases of disease, and the epidemic potential remains high (Staples et al., 2009). Although the mortality rate of CHIKV disease is not high, 0.1%, the disease manifestations lead to chronic disability have considerable implications including a substantial impact on quality of life for infected patients as well as considerable economic and community consequences (Couturier et al., 2012, Schilte et al., 2013). For this reason, the search for compounds, particularly licensed drug with antiviral potential, is a research priority. Concomitantly, the understanding of the intracellular mechanisms involved in vector-arbovirus-vertebrate host interaction is a prerequisite to the elaboration of an efficious therapy.

Imipramine Inhibits Chikungunya Virus Replication in Human Skin Fibroblasts through Interference with Intracellular Cholesterol Trafficking

Various scientists attempt for the development of therapeutics and drugs treatment of CHIKV infection. These regimens are various chemical compounds, RNA based inhibitors and inhibitors targeting cellular factors (Parashar and Cherian, 2014). Previous reports have suggested chloroquinone has antiviral activity against many viruses including CHIKV (Chopra et al., 2014). Ribavirin alone or in combination with INF-α2b has a potent antiviral activity against CHIKV (Briolant et al., 2004). Similarly, Ribavirin in combination with doxycycline showed a synergistic effect on the *in vitro* and *in vivo* inhibition of CHIKV (Rothan et al., 2015). Favipiravir, a broad-spectrum antiviral drug, was shown to inhibit viral replication in both cell cultures and mice (Delang et al., 2014). However, until now, there is no licensed drug specific for this disease, the extension for searching antiviral compounds would be useful.

In the present study, we have evaluated whether drugs that interfere with intracellular cholesterol transport have the capacity to inhibit CHIKV replication in epidermal fibroblasts, a major target cell for viral entry into human host. The results show an antiviral activity of the class II cationic amphiphilic compounds U18666A and imipramine. Both drugs, can block the cellular trafficking of cholesterol in the LE/Ls compartment, the site of viral membrane fusion and cytoplasmic escape, and induce a phenotype in human fibroblasts reminiscent to that observed in Niemann-Pick type C disease.

Interestingly, imipramine strongly inhibits the replication not only of CHIKV, but also of several members of the *Flaviviridae* family, including ZIKV, WNV and DENV.

U18666A was reported to inhibit distinct steps of virus replication such as the entry of DENV and Ebola virus (Carette et al., 2011, Poh et al., 2012, Shoemaker et al., 2013) and the assembly of HIV-1 particles (Shoemaker et al., 2013, Tang et al., 2009). Our results agree with these findings and show that U18666A strongly inhibits, in a dose-dependent manner, the replication of CHIKV in human fibroblast cells. The effect also be found in imipramine, an FDA-approved antidepressant drug (Aboukhatwa and Luo, 2011) and is consistent with the results from a recent study showing that imipramine inhibits Ebola virus production of in human umbilical vein epithelial cells (Herbert et al., 2015). Moreover, The drug interferes with obvious steps of the infectious cycle requiring cholesterol, such as entry and fusion steps. Indeed, host membrane cholesterol is generally recognized as a key factor for the unmasking of the fusion peptide in alphavirus including that of CHIKV (Bernard et al., 2010, Hoornweg et al., 2016, Kielian et al., 2010, Teissier and Pécheur, 2007).

Together, imipramine was shown to be a potent *in vitro* antiviral compound for CHIKV as well as other Flaviviruses. Additionally, since imipramine exerted its antiviral effects in all the three modes of treatment (pre-treatment, concurrent, and post-treatment), it was suggested that it has both prophylactic and therapeutic potentials. However, further investigations are needed (i) to expand the spectrum of imipramine antiviral activity to other arboviruses (ii) to fully understand the mechanism of imipramine in each step of viral replication (iii) to study the antiviral activity and side effect of imipramine in an *in vivo* model. These knowledge could help to elucidate the antiviral mechanism induced by the drug and/or others member of class II cationic amphiphilic compounds as well as other possible effects beyond to the antiviral activity that can help to relief the pathogenesis of arboviruses.

SAMHD1 enhances Chikungunya and Zika virus replication in human skin fibroblasts

To gain deeper insight into the biological processes and cellular regulatory proteins affecting viral transmission cycle, the study of differential expression of host proteins during viral infection is needed. Recently, several studies have evaluated the proteomic profile of human host cells upon CHIKV infection (Abere et al., 2012, Issac et al., 2014, Thio et al., 2013, Wikan et al., 2014). However, until now, there are no reports pertaining to infection with ZIKV, another arbovirus member that poses a serious global public health concern

(Hamel et al., 2016). Notably, the differential regulation of proteins in CHIKV and ZIKV infected human skin cells, the primary site of infection, is still unknown.

Therefore, in this study, we have determined the global proteomic profile of CHIKV and ZIKV infected human skin fibroblasts, using Stable Isotope Labeling by Amino acids in Cell culture (SILAC) based LC-MS/MS analysis. We found that nine proteins including MX1, IFIT1, IFIT3, ISG15, OAS3, DDX58, STAT1, EIF2AK2 and SAMHD1, which are significantly up-regulated in human skin fibroblasts infected with CHIKV or ZIKV.

Important arm of the host immune system to defense against viral infection is innate immunity, which establishes an antiviral state in cells by producing type I IFN and subsequently up-regulation of hundreds of ISGs. IFITs are ISG that have a broad spectrum of antiviral defenses. The inhibiting activities of IFITs have been documented such as the blocking of translation initiation factor (Hui et al., 2003), the binding of uncapped or incompletely capped viral RNA (Habjan et al., 2013, Pichlmair et al., 2011, Schmeisser et al., 2010) and the sequestration of viral proteins or RNAs in the cytoplasm (Saikia et al., 2010, Terenzi et al., 2008). Two individual research groups have shown that IFITs have specific antiviral functions versus human parainfluenza virus type 3 and 5 (Andrejeva et al., 2013, Rabbani et al., 2016). IFIT1 has documented to interferes with translation and replication of alphavirus genomes (Reynaud et al., 2015). Recently, Frumence *et al.* reported that ZIKV infection can induces IFIT1 and 2 genes expression in human lung epithelial A549 cells (Frumence et al., 2016). In the present study, our results confirm and extend these observations by showing that the forced expression of IFIT1, IFIT2 or IFIT3 in human fibroblasts completely inhibited the replication of CHIKV which was restored when the genes encoding these IFITs were individually knocked-down in these cells. Nevertheless, in contrast to its strong inhibitory effect on CHIKV replication, IFIT2 does not be involved in the inhibition of ZIKV replication in human skin fibroblasts.

The different antiviral function of IFIT2 in CHIKV and ZIKV infection may be due to the type of viruses and IFITs structure. Infact, different IFIT family members have distinct numbers of tetratricopeptide repeat structures, which may dictate specific functions (D'Andrea and Regan, 2003). Together, the results suggest that IFIT1 and 3 are involved in the replication of these viruses.

Finding of the inhibitory properties of IFITs proteins are value, as they could serve as targets for controlling of CHIKV and ZIKV replication in mammalian hosts. However, the molecular mechanism and the cooperative action of IFITs involved in viral infection still need to be determined.

Furthermore, the blocking capability of these proteins in the transmission of other arboviruses has to be investigated. Crystal structure of these proteins might allow us to design compound(s) capable of mimic its activity and hence to, at least, reduce the viral charge.

SAMHD1 is a protein reported to function as the restriction factor of HIV-1 in resting cells. It has been shown to be degraded by the accessory factor Vpx, which is encoded by HIV-2 and the closely related simian immunodeficiency virus strains, through a proteasome-dependent mechanism (Berger et al., 2011, Hrecka et al., 2011, Laguette et al., 2011). SAMHD1 has both dNTPase, in nucleus, and RNase activities, in cytoplasm. Due to these activities, it has been reported to decreases intracellular dNTPs pool to under the levels required for retroviral replication and to restrict HIV-1 through the RNA digestion (Choi et al., 2015, Lahouassa et al., 2012, Ryoo et al., 2014). Recently, it was suggested that SAMHD1 can also restrict Hepatitis B virus through its dNTPase activity (Chen et al., 2014, Sommer et al., 2016). The antiviral activity of SAMHD1 is regulated through phosphorylation of Thr592 by cyclin-dependent kinase 1 or 2 (CDK1 or 2) (Cribier et al., 2013, White et al., 2013a). Despite these findings, no information is available regarding the role of SAMHD1 during arbovirus infection.

In this study, we found that degradation of SAMHD1 by Vpx/VLP-mediated causes a decrease in CHIKV and ZIKV replication and production. These findings are supported by the observation that overexpression of endogenous SAMHD1 in human skin fibroblasts and U937 cells enhances the viral replication of both CHIKV and ZIKV, while knockdown of SAMHD1 has the opposite effect. Discrepancy of our work and the previous studies that have underscored the role of SAMHD1 as a restriction factor able to inhibit the replication of reverse transcription-required viruses (Chen et al., 2014, Hollenbaugh et al., 2013, Kim et al., 2013, Sommer et al., 2016, White et al., 2013a, Zhao et al., 2013) may be because of the type of viruses. Indeed, unlike HIV-1, CHIKV and ZIKV do not have the reverse transcription step for their replication.

Collectively, these data provide novel evidence on the role of SAMHD1 in arbovirus infection of human skin cells. However, the precise molecular mechanisms by which SAMHD1 increases CHIKV and ZIKV replication remains to be investigated. It has been reported that phosphorylation of SAMHD1 at residue threonine 592 (Thr592) by cyclin A2/CDK1 is a key regulatory mechanism of its antiviral activity in HIV-1 (Cribier et al., 2013).

It should be interesting to investigate whether Thr592 residue is also important for pro-viral activity of SAMHD1. Finally, since it has been reported that type I IFN pathway influences the expression of SAMHD1 via IRF3 (Yang et al., 2016), the regulation of SAMHD1 by this pathway in the context of arboviruses infection would also be interesting to study.

Aedes Aegypti Saliva Enhances Chikungunya Virus Replication in Human Skin Fibroblasts

CHIKV transmission is initiated through the bite of an infected female *Aedes* mosquito when the virus is injected, together with mosquito saliva, into the skin of the human host. Mosquito saliva contains various pharmacologically active molecules that contribute to successful blood feeding by inhibiting host hemostasis, inflammation and immune responses (Wichit et al., 2016). Several studies on DENV and WNV reported the role of mosquito saliva in the induction of viral replication (Styer et al., 2011, Surasombatpattana et al., 2012, Wichit et al., 2016). However, the study employed the mosquito probing then follow by mosquito inoculation in mouse model which does not similar to human hosts.

In the present study, we have determined the effect of *Ae. aegypti* fresh collected saliva on CHIKV replication in human skin fibroblasts which mimics the situations that occur during natural transmission. The viral replication was significant increased in fibroblasts infected with CHIKV in the presence of the saliva, as compared with that in cells infected with virus alone.

These data support previous observations showing that *Ae. aegypti* saliva has an important role in the inhibition of an antiviral immune response by the host by creating an environment that favors the replication of CHIKV (Agarwal et al., 2016). We have also studied immunological factors that may support the induction of CHIKV by mosquito saliva. As type I IFNs are known to elicites by CHIKV infection resulting in the production of other pro-inflammatory cytokines (Lum and Ng, 2015). We have hypothesized that saliva may supress the IFN pathway in order to benefit the viral production. As expected, in our study, CHIKV-infected cells in the presence of mosquito saliva were found to significantly reduce the expression level of many type I IFN-responsive genes, when compared to those of the infected cells without mosquito saliva.

To our knowledge, this is the first study showing the importance of *Ae. aegypti* saliva in promoting CHIKV infection via the reduction of genes involved in type I IFN secretion by infected human skin fibroblasts. Nontheless, elucidation of the molecular mechanisms underlying this process needs to be further investigated. In order to mimic the natural infection in human skin compartment, it should be interesting to evaluate the effects of mosquito saliva in a global model such as human skin biopsy.

By modulating the immune response at the bite site, saliva could thus benefit the virus and facilitate its transmission to other target cells. Indeed, the saliva contains numerous known, as well as yet to be determined, proteins that, through their capacity to modulate the host immune response, are essential for optimal transmission of CHIKV, DENV and other arboviruses (Agarwal et al., 2016, Schneider and Higgs, 2008, Surasombatpattana et al., 2012, Thangamani et al., 2010). Following a functional genomic and proteomic analysis of salivary glands of female *Ae. aegypti* mosquitoes, our group identified four additional proteins, AT: Q1HRTV7_AEDAE, AD: Q179D4_AEDAE, 34kD: Q1HRW0_AEDAE and VA: Q8T9U5_AEDAE, that are abundantly present in mosquito saliva (Luplertlop et al., 2011, Wasinpiyamongkol et al., 2010). It should be interesting to investigate the implication and mechanism of the four identified proteins in CHIKV infection. These molecules could serve as potential targets for the controlling CHIKV replication in mammalian hosts.

The work presented in this thesis resulted in an accepted publication which I am the first author. I am also the first author of one publication under revision and one publication in preparation. During my thesis, I also participated in other research developed in Dorothée Missé's team (presented below).

List of publications

1. Imipramine Inhibits Chikungunya Virus Replication in Human Skin Fibroblasts through Interference with Intracellular Cholesterol Trafficking

   Sineewanlaya Wichit*, Rodolphe Hamel, Eric Bernard, Loïc Talignani, Fodé Diop, Pauline Ferraris, Florian Liegeois, Peeraya Ekchariyawat, Natthanej Luplertlop, Pornapat Surasombatpattana, Frédéric Thomas, Andres Merits, Valérie Choumet, Pierre Roques, Hans Yssel, Laurence Briant and Dorothée Missé*

   Sci Rep. 2017, Jun 9;7(1):3145. doi: 10.1038/s41598-017-03316-5

2. *Aedes Aegypti* Saliva Enhances Chikungunya Virus Replication in Human Skin Fibroblasts

   ***Sineewanlaya Wichit**, Fodé Diop, Rodolphe Hamel, Loïc Talignani, Pauline Ferraris, Florian Liegeois, Hans Yssel and Dorothée Missé*

   J Invest Dermatol. 2017, in Revision

3. SAMHD1 enhances Chikungunya and Zika virus replication in human skin fibroblasts

   ***Sineewanlaya Wichit**, Rodolphe Hamel, Andreas Zanzoni, Fodé Diop, Alexandra Cribier, Loïc Talignani, Abibatou Diack, Pauline Ferraris, Florian Liegeois, Natthanej Luplertlop, Andres Merits, Hans Yssel, Monsef Benkirane and Dorothée Missé*

   Manuscript in preparation

4. The effects of mosquito saliva on dengue virus infectivity in humans.

   ***Sineewanlaya Wichit**, Ferraris P, Choumet V, Missé D.*

   Curr Opin Virol. 2016, 21:139-145.

5. Zika virus causes supernumerary foci with centriolar proteins and impaired spindle positioning

   *Benita Wolf, **Sineewanlaya Wichit**†, Fode´ Diop†, Pauline Ferraris†, Coralie Busso, Dorothée Missé and Pierre Gönczy*

   Open Biol. 2017 Jan; 7(1): 160231.

6. African and Asian Zika virus strains differentially induce early antiviral responses in primary human astrocytes

   *Rodolphe Hamel, Pauline Ferraris, **Sineewanlaya Wichit** , Fodé Diop , Loïc Talignani , Julien Pompon , Déborah Garcia , Florian Liégeois , Amadou A. Sall , Hans Yssel , Dorothée Missé*

   Infect Genet Evol. 2017, 49: 134–137.

7. Zika virus: epidemiology, clinical features and host-virus interactions.

   *Hamel R, Liégeois F, **Wichit S**, Pompon J, Diop F, Talignani L, Thomas F, Desprès P, Yssel H, Missé D.*

   Microbes Infect. 2016, 18(7-8): 441-9

8. Biology of Zika Virus Infection in Human Skin Cells.

*Hamel R, **Wichit S**[#], Dejarnac O[#], Ekchariyawat P, Neyret A, Luplertlop N, Perera-Lecoin M, Surasombatpattana P, Talignani L, Thomas F, Cao-Lormeau VM, Choumet V, Briant L, Desprès P, Amara A, Yssel H, Missé D.*

J Virol. 2015, 89(17): 8880-96.

9. Inflammasome signaling pathways exert antiviral effect against Chikungunya virus human dermal fibroblasts.

*Ekchariyawat P, Hamel R, Bernard E, **Wichit S**, Surasombatpattana P, Talignani L,Thomas F, Choumet V, Yssel H, Desprès P, Briant L, Missé D.*

Infect Genet Evol. 2015, 32:401-8.

CHAPTER VI:

Résumé des travaux en français

Rôle du cholestérol, de la protéine SAMHD1 et de la salive d'*Aedes aegypti* dans l'infection des cellules cutanées par le virus chikungunya

I-CONTEXTE

Durant ces vingt dernières années on a assisté à une augmentation considérable, dans certaines régions du monde, des épidémies dues à des arbovirus. Des épidémies de dengue, de zika et de chikungunya durant ces dix dernières années soulignent l'importance en santé publique de ces arboviroses (Cao-Lormeau, 2016). Les facteurs influant sur l'expansion des arbovirus sont divers. Ils relèvent entre autres des activités humaines, de l'environnement, des conditions climatiques. Les facteurs écologiques et urbains jouent sur les interactions entre les moustiques et les hommes ainsi que les réservoirs animaux. Un des facteurs d'émergence de ces virus est la présence de vecteurs compétents, tels que les moustiques *Aedes (Ae.) aegypti* et *Aedes albopictus*, dans différentes régions du monde (Figure 1). Des adaptations virales été décrites comme un changement de nucléotide du virus chikungunya (CHIKV) lors de l'épidémie de La Réunion, responsable de sa meilleure adaptation à *Ae. Albopictus* (de Lamballerie et al., 2008). Cette mutation est peut-être pour partie responsable de l'explosion épidémique qu'il y a eu dans cette région du monde. Le nombre de cas de personnes touchées par des infections arbovirales est en constante augmentation dans les régions endémiques. On note également la présence nouvelle de ces virus dans différentes régions (Mayer et al., 2017).

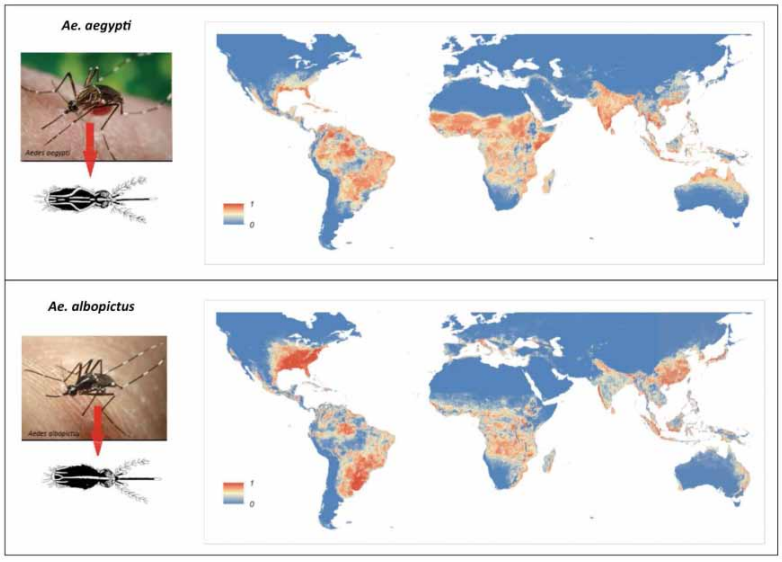

Figure 1. Global distribution of *Ae. aegypti* and *Ae. albopictus* and its morphology. Adapted from (Kraemer et al., 2015).

Lorsque l'on regarde la distribution géographique du virus de la dengue (DENV), du CHIKV et du virus Zika (ZIKV) durant ces dix dernières années, l'expansion du DENV est un processus continu. En revanche, l'émergence du CHIKV dans l'Océan Indien puis dans les caraïbes et celle du ZIKV dans le pacifique ont dramatiquement modifié la carte de distribution de ces virus.

Bien que des modes de transmissions alternatifs aient été décris dans la littérature pour certains de ces virus, le mode de transmission principal entre les hôtes vertébrés se fait par l'intermédiaire d'arthropodes hématophages, tels que les tiques, les phlébotomes ou les moustiques (Choumet and Desprès, 2015, D'Ortenzio et al., 2016). Les moustiques du genre *Aedes* sont très répandus et se retrouvent actuellement dans tous les continents, y compris l'Amérique du Nord et l'Europe, avec 3 milliards de personnes vivants dans des régions infestés par ces moustiques (Kraemer et al., 2015). La transmission du CHIKV se fait par piqûre d'un moustique du genre *Aedes* (Briant et al., 2014). Lors de cette piqûre de moustique, une grande partie du contenu des glandes salivaires est relarguée dans le milieu extravasculaire dans le compartiment cutané de l'hôte vertébré. Les glandes salivaires de moustiques secrètent une diverses protéines ayant des propriétés pharmacologiques variées (Wichit et al., 2016). Certaines d'entre elles permettent une bonne prise du repas sanguin en inhibant la voie de l'hémostase, l'inflammation et la réponse immune de l'hôte (Fontaine et al., 2011). De plus, il a été démontré que la salive de moustique inhibe significativement l'expression de gènes impliqués dans la réponse inflammatoire et la production de chimiokines, créant ainsi un environnement qui favorise la réplication du CHIKV (Agarwal et al., 2016).

II. OBJECTIFS PRINCIPAUX DE LA THÈSE

Malgré l'expansion des arbovirus tels que le CHIKV et le ZIKV, les moyens de contrôle traditionnels des arbovirus, tels la vaccination, les antiviraux, ou les moyens de contrôle des moustiques, tels les insecticides, sont la plupart du temps inexistants ou restreints dans leur champs d'application. Pour cette raison, la recherche de composés ayant des propriétés antivirales est une priorité en santé publique. Des recherches doivent également être entreprises afin de comprendre les mécanismes moléculaires mis en jeu lors de l'infection des cellules cibles par ces virus en prenant également en compte la triade virus-vecteur-hôte vertébré.

L'objectif principal de cette thèse était de comprendre le rôle du cholestérol, de la protéine SAMHD1 et de la salive du vecteur *Aedes aegypti* dans la transmission du virus Chikungunya.

Les objectifs spécifiques sont :

- D'évaluer le potentiel antiviral de molécules qui interfèrent avec le transport du cholestérol dans un modèle d'infection de fibroblastes cutanés par le CHIKV
- D'évaluer le rôle des protéines impliquées dans la réplication du CHIKV et du ZIKV dans les fibroblastes cutanés
- D'étudier l'impact de la salive du moustique *Aedes aegypti* dans l'infection des fibroblastes cutanés par le CHIKV

III. RÉSULTATS

III-I. L'imipramine inhibe la réplication du virus chikungunya dans les fibroblastes cutanés en interférent avec le transport intracellulaire du cholestérol.

Le cholestérol membranaire est généralement reconnu comme un facteur clé pour l'exposition des peptides de fusion des glycoprotéines d'enveloppe de classe II (Teissier and Pécheur, 2007) y compris celle du CHIKV (Bernard et al., 2010, Hoornweg et al., 2016). Dans cette étude nous avons évalué le potential antiviral contre le CHIKV, de deux composés (U18666A et l'imipramine) connus pour bloquer le transport intracellulaire du cholestérol. Nous avons choisi comme modèle cellulaire les fibroblastes cutanés car ils représentent un des premiers type cellulaire que le virus rencontre lors de l'injection du virus par le moustique dans la peau (Ekchariyawat et al., 2015). Nous avons dans un premier temps, démontré que ces deux composés étaient capable de bloquer le transport du cholestérol dans les endosomes tardifs/lysosomes dans les fibroblastes. L'endolysosome est un site capital où se déroule la fusion entre les membranes cellulaires et virales. Nous avons observé une réduction drastique de la réplication et la production du CHIKV dans les fibroblastes exposés au composé U18666A ou à l'imipramine et ceci de manière dose dépendante (Figure 2). Par la suite, nous avons focalisé nos travaux sur l'impact de l'imipramine dans les étapes du cycle de réplication du CHIKV. L'imipramine a été choisi car c'est une molécule déjà mise sur le marché et qui est utilisée comme antidépresseurs (Aboukhatwa and Luo, 2011). Nos résultats montrent que cette dernière agit au niveau de l'entrée viral/fusion mais aussi dans les étapes

postérieures à la fusion. Il est important de souligner que nous n'avons pas observé de cytotoxicité avec les doses d'imipramine utilisées.

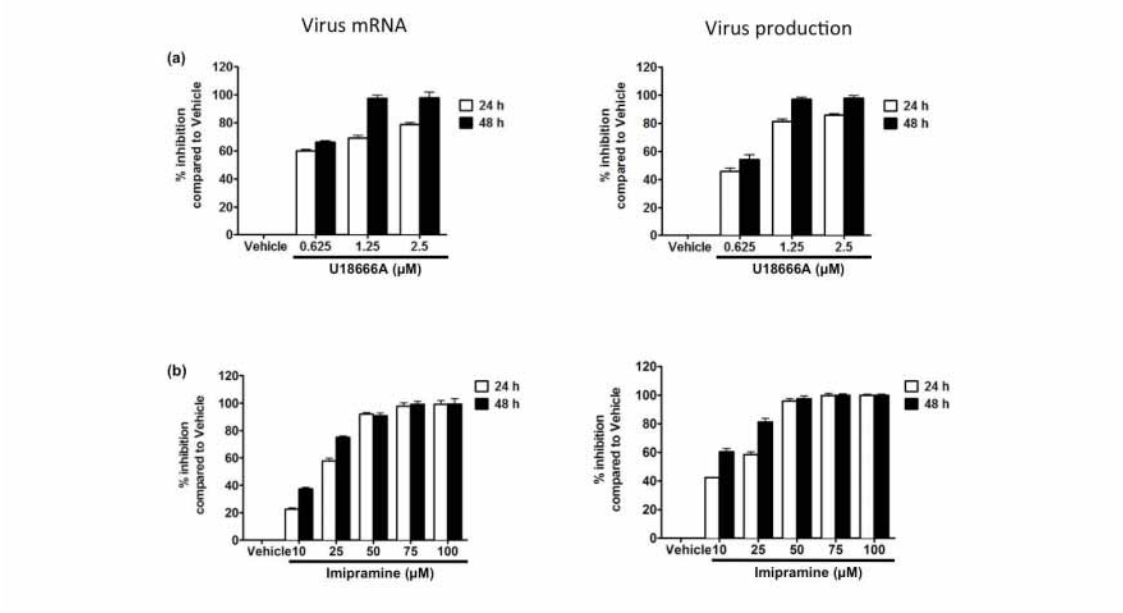

Figure 2. Human skin fibroblasts were treated with vehicle, U18666A (a) or imipramine (b) before infection with CHIKV La Réunion strain (MOI 1). After 24 and 48 h, virus RNA and infectious virus production were measured by real time RT-PCR and plaque assay, respectively. Inhibition at the presence of vehicle was set as "0" and percentage of inhibition at the presence of inhibitor was calculated using formula [1-(I/V)]*100 where V and I designate experimental values (RNA copy numbers or plaque numbers) at the presence of vehicle and inhibitor, respectively. The data represent mean ± SD from three independent experiments.

Nous avons étendu nos travaux à d'autres arbovirus et démontré que l'imipramine inhibe la replication du ZIKV, du DENV et du virus West Nile (Figure 3). Les fibroblastes mis en contact avec l'imipramine ont un phénotype similaire à celui des fibroblastes de patients atteints de la maladie de Niemann-Pick de type-C (NPC). Cette maladie est une pathologie neurologique progressive rare d'origine génétique qui est due au dysfonctionement d'une ou des deux proteins endosomales NPC1 et NPC2, résultant en une accumulation de cholestérol intracellulaire. Nous avons observé une inhibition significative du pourcentage de fibroblastes de différents patients NPC infectés par le CHIKV comparés à l'infection de cellules provenant de donneurs sains. Cette observation a été confirmée par une forte réduction de la production de particules virales dans les cellules de patients NPC. Nos résultats démontrent que la déficience en NPC1 et NPC2 a un impact dans les étapes d'entrée et/ou de fusion dans le cycle de replication du CHIKV.

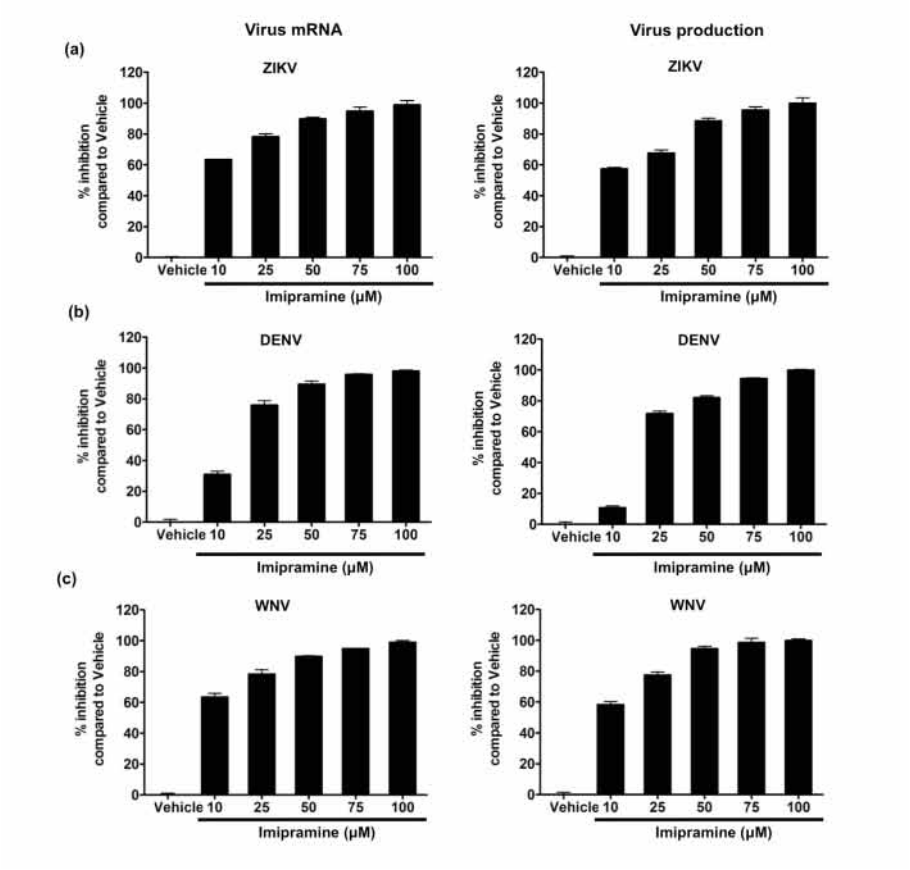

Figure 3. Human skin fibroblasts were treated with vehicle or imipramine before infection with ZIKV (a), DENV (b) or WNV (c). After 48 h, virus RNA and infectious virus production were measured by real time RT-PCR and plaque assay, respectively. Inhibition at the presence of vehicle was set as "0" and percentage of inhibition at the presence of inhibitor was calculated using formula [1-(I/V)]*100 where V and I designate experimental values (RNA copy numbers or plaque numbers) at the presence of vehicle and inhibitor, respectively. The data represent mean ± SD from three independent experiments.

D'autres équipes ont par le passé montré que le composé U18666A inhibe l'entrée du DENV et du virus Ebola (Carette et al., 2011, Poh et al., 2012, Shoemaker et al., 2013) et bloque l'assemblage des particules HIV-1 (Shoemaker et al., 2013, Tang et al., 2009). De plus, des travaux récents ont montré que l'imipramine inhibe la production du virus Ebola dans les cellules endothéliales (Herbert et al., 2015). Tous ces travaux ainsi que ceux obtenus au cours de cette thèse souligne l'importance du cholestérol dans le cycle de réplication de différents virus et montre l'importance de s'intéresser aux composés qui ciblent son trafic intracellulaire. Cependant, des travaux supplémentaires doivent être menés, notamment des études *in vivo* afin d'évaluer l'efficacité antiviral de ce type de molécules et les potentiels effets secondaires qu'ils pourraient induire. Il serait aussi intéressant de déterminer avec précision les différentes étapes, en dehors de la fusion, qui sont impactées par l'imipramine.

III-II. La protéine SAMHD1 augmente la réplication des virus Chikungunya et Zika dans les fibroblastes humains

Les interactions entre des virus et leurs cellules cibles par des analyses protéomiques à grande échelle pourraient fournir des informations précieuses sur les protéines cellulaires pouvant jouer un rôle dans la réplication et la production virale. Actuellement aucune étude protéomique n'a été réalisée sur des cellules cutanées (site d'entré des arbovirus) infectées par des arbovirus. C'est dans cette optique que nous avons réalisé en utilisant la méthode SILAC (stable isotope labelling by amino acids in cell culture) des analyses protéomiques comparatives entre des fibroblastes cutanés sains ou infectés soit par le CHIKV ou par le ZIKV. Il est important de souligner que récemment nous avons montré que ces cellules étaient permissives au ZIKV (Hamel et al., 2015). Notre étude a montré que plusieurs protéines étaient communément régulées par le CHIKV et le ZIKV (Figure 4).

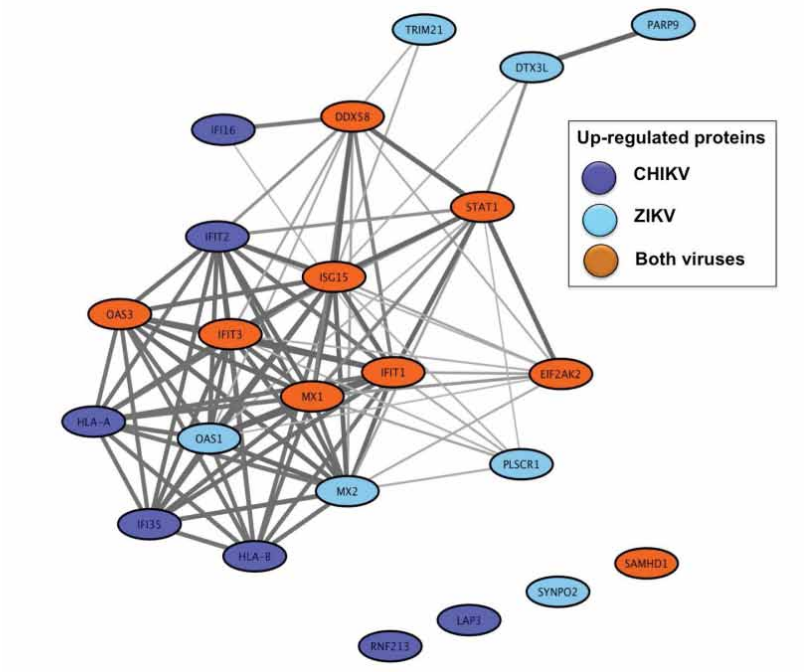

Figure 4. Network representation of significantly up-regulated proteins in human skin fibroblast infected cells. Merged functional interaction network among all significantly up-regulated proteins in both CHIKV and ZIKV infected cells. Proteins are represented as nodes and functional relationships by edges. The thickness of edges is proportional to the confidence level of the functional relationship.

Ces protéines (MX1, IFIT1, IFIT3, ISG15, DDX58, STAT1, OAS3, EIF2AK2 et SAMHD1) sont surexprimées lors de l'infection et sont essentiellement décrites comme impliquées dans la défense de l'hôte. Nous avons focalisé nos travaux sur la compréhension du rôle de la protéine SAMHD1 dans l'infection des fibroblastes par le CHIKV et le ZIKV. Cette protéine a été décrite comme un facteur de restriction du VIH-1 (Berger et al., 2011, Hrecka et al., 2011, Laguette et al., 2011). Elle possède une activité dNTPase qui diminue le pool intracellulaire de dNTPs essentiel pour la réplication des rétrovirus (Lahouassa et al., 2012, St Gelais et al., 2012). Son activité antiviral est régulée par sa phosphorylation sur le résidu Thr592 qui rend la protéine incapable d'inhiber les rétrovirus (Cribier et al., 2013, White et al., 2013a). Une mutation de la protéine sur ce résidu empêche sa phosphorylation. Il a aussi été démontré que la protéine Vpx du VIH-2 était capable de dégrader SAMHD1 (Laguette et al., 2011). Sachant que les arbovirus CHIKV et ZIKV n'ont pas besoin de dNTP pour accomplir leur cycle de réplication, nous avons voulu élucider le rôle de la protéine SAMHD1 dans l'infection des fibroblastes par ces virus. Nous avons démontré qu'une surexpression de SAMHD1 dans les fibroblastes cutanés entraîne une augmentation significative de la réplication des deux arbovirus (Figure 5A and B). A contrario, la transduction des cellules par des VLPs (Viral like particles) contenant la protéine VPx inhibe la réplication du CHIKV et du ZIKV (Figure 5C and D). De plus, la suppression de l'expression de SAMHD1 résulte en une inhibition de la production de particules virales. Dans l'ensemble ces résultats démontrent que SAMHD1 est un facteur qui augmente la réplication du CHIKV et ZIKV.

Il est important de noter que ce travail révèle un nouveau mode d'action de la protéine SAMHD1. En effet, les études précédentes basées sur des virus nécessitant une transcription inverse pour accomplir leur cycle de réplication ont toujours décrit cette protéine comme un facteur de restriction viral (Chen et al., 2014, Hollenbaugh et al., 2013, Kim et al., 2013, Sommer et al., 2016, White et al., 2013a, Zhao et al., 2013). Des études supplémentaires restent cependant à réaliser afin de décrypter les mécanismes moléculaires permettant à la protéine SAMHD1 d'augmenter la réplication des arbovirus CHIKV et ZIKV. De plus, Il serait intéressant de voir si la forme phosphorylée de SAMHD 1 joue un rôle dans l'infection de ces deux arbovirus.

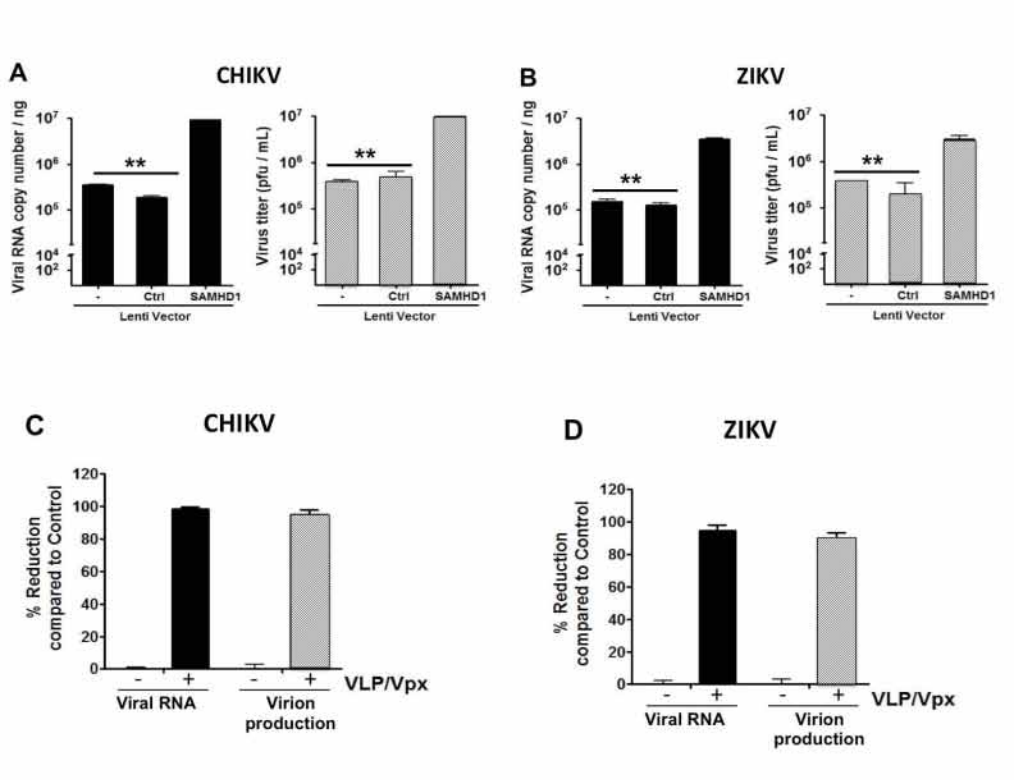

Figure 5. (A and B) HFF1 normal (-), stably expressing control (Ctrl) or SAMHD1 cells were exposed to CHIKV or ZIKV for 48 h then virus RNA was quantified by real time RT-PCR (black bar) and plaque assay (gray bar), respectively. (C and D) Vpx non treated or treated cells were exposed to CHIKV or ZIKV. After 48 h, virus RNA and infectious virus production were measured by real time RT-PCR (black bar) and plaque assay (gray bar), respectively. Reduction at the presence of control (no Vpx) was set as "0" and percentage of reduction at the presence of Vpx was calculated using formula [1-(R/C)]*100 where C and R designate experimental values (RNA copy numbers or plaque numbers) at the presence of control and Vpx, respectively.

III-III. La salive d'Aedes Aegypti augmente la Réplication du virus chikungunya dans les fibroblastes cutanés

Lors d'un repas sanguin, les insectes hématophages sont soumis à une réponse défensive du vertébré sur lequel il prélève le sang et notamment la coagulation et la réponse immune. Il a été décrit que la salive injectée par le moustique, lors de la piqûre, possède des substances vasodilatatrices, anti-coagulantes et anti-plaquettaires qui permettent de faciliter la prise du repas sanguin (Wichit et al., 2016 ; Patramool et Coll., 2013 ; Bradley et Coll., 2008 ; Titus et Coll., 2006).

Plusieurs études ont montré que la salive des arthropodes vecteurs de maladies infectieuses, lorsqu'elle est injectée à l'hôte vertébré au moment du repas sanguin, est susceptible de jouer un rôle crucial dans la capacité du vecteur à transmettre le pathogène qu'elle contient (Surasombatpattana et al., 2012; Kamhawi et Coll., 2000; Schneider et Coll., 2008; Titus et Coll., 2006; Billingsley et Coll., 2006; Schneider et Coll., 2010; Le Coupanec et Coll., 2013). Il a été décrit que la salive d'*Aede*s était capable d'augmenter l'infection et la réplication de différents virus, tels que le Cache Valley virus (Edwards et Coll., 1998), le virus de La Crosse (Osorio et Coll.,1996, le virus de la stomatite vésiculaire (Limesand et Coll., 2003), le virus de la Fièvre de la Vallée du Rift (Le Coupanec et Coll., 2013) et le virus de la dengue (Surasombatpattana et al., 2012 ; Surasombatpattana et al., 2014). Inversement, d'autres études ont suggéré que l'environnement pro-inflammatoire, induit par des composants de la salive d'*Aedes,* pourrait protéger l'hôte des agents pathogènes (Kamhawi et Coll., 2000). Une étude récente a montré que des souris exposées à une piqûre du moustique *Aedes aegypti* puis inoculées par du CHIKV présentaient une virémie dix fois plus importante que des souris uniquement infectées par ce virus (Agarwal et al., 2016). Sachant que les fibroblastes cutanés sont un des types cellulaires privilégiés du CHIKV, nous avons voulu savoir si la salive d'*Aedes aegypti* était capable (1) d'augmenter l'infection de ces cellules par le CHIKV et (2) de moduler la réponse immune antivirale. Notre étude a pu démontrer qu'en présence de salive on avait une augmentation d'environ 2 logs de la quantité d'ARN viral intracellulaire à 48h après infection (Figure 6). Nous avons également observé une augmentation de la production de particules virales dans les mêmes conditions.

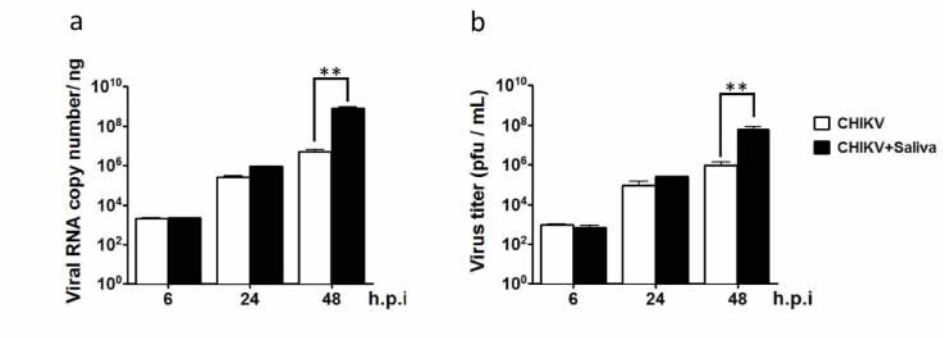

Figure 6. *Ae. aegypti* saliva enhances CHIKV replication in human skin fibroblasts. Cells were infected with CHIKV at a multiplicity of infection of 1 in the presence or absence of 1 µg/ml *Ae. aegypti* saliva. After 6, 24 and 48 hours, intracellular viral RNA and infectious virus production were quantified by (a) real-time quantitative RT-PCR and (b) plaque assay, respectively.

Sachant que la réponse interféron représente la première ligne de défense contre les infections virales, et qu'il a été décrit que le CHIKV induit la production des interférons de type I (Lum and Ng, 2015), nous avons concentrer nos efforts sur cette voie. Nous avons démontré par une analyse transcriptomique une diminution importance de l'expression de plusieurs gènes induits par l'interféron dans les cellules infectées en présence de la salive d'*Aedes aegypti* (Figure 7). En modulant l'immunité locale dans laquelle le virus va être transmis, la salive pourrait ainsi avantager le virus et lui permettre d'atteindre plus facilement ses autres cellules cibles. Par la suite, il serait intéressant d'identifier la ou les protéine(s) salivaires impliquée(s) dans la modulation de la voie interféron dans les fibroblastes. Ces protéines pourraient être des cibles potentielles pour éviter la dissémination du CHIKV.

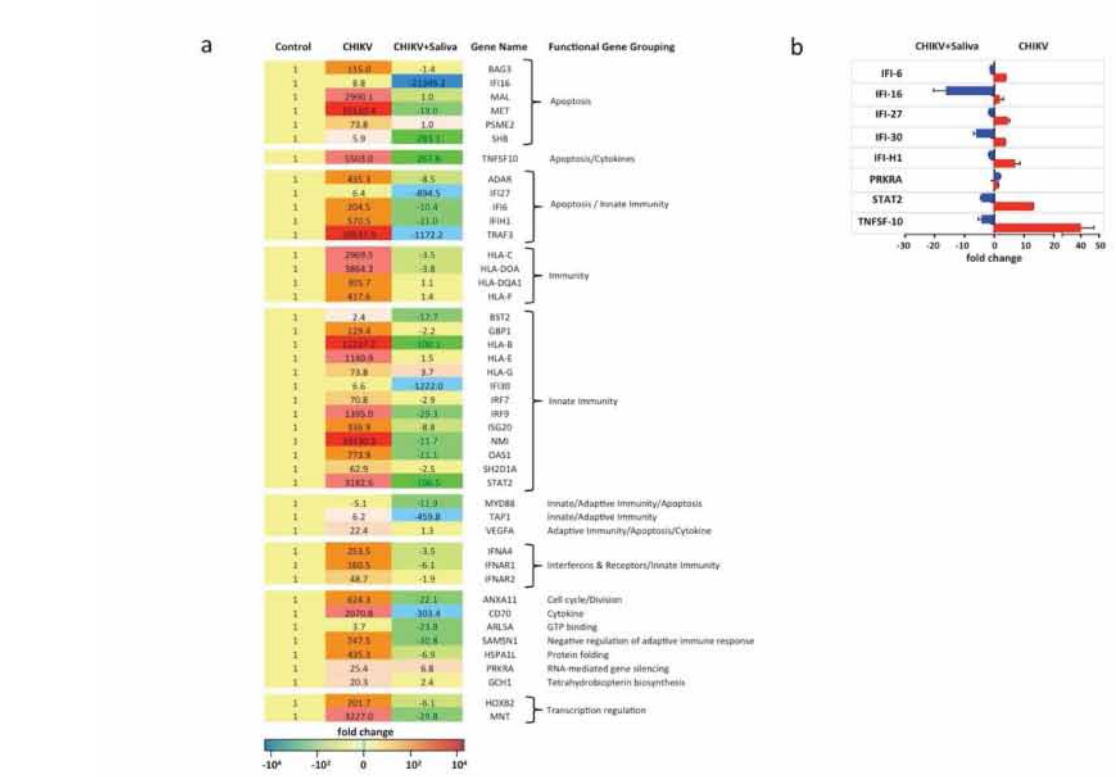

Figure 7. Expression of genes involved in human type I interferon response regulation by CHIKV in human skin fibroblasts. Cells were exposed to CHIKV at a multiplicity of infection of 1 in the presence or absence of 1 µg/ml *Ae. aegypti* saliva. (a) The expression of significantly up- or down-regulated genes involved in the human type I interferon response was measured by PCR array. (b) mRNA levels of the indicated gene were validated by real-time quantitative RT-PCR. Results are expressed as fold change of transcripts in CHIKV-infected human skin fibroblasts in the presence or absence of saliva, relative to those in mock-infected cells (Control).

IV. Conclusion

L'ensemble de ces travaux de thèse a permis de contribuer aux connaissances sur la transmission du CHIKV. Ce travail a mis en évidence que des composés comme l'imipramine ciblant le trafic intracellulaire du cholestérol pouvaient avoir une activité antivirale contre ce virus mais aussi d'autres arbovirus. Il a également mis en lumière un nouveau rôle que pourrait jouer la protéine SAMHD1 dans la transmission virus. Enfin, il démontre l'importance de prendre en compte la triade virus-vecteur-hôte vertébré dans la transmission des arbovirus.

La valorisation de cette thèse a donné lieu à une publication acceptée et une revue dont je suis le premier auteur. Une publication en cours de révision et une publication en préparation dont je suis également le premier auteur. Au cours de ma thèse, j'ai également participé à d'autres travaux de recherche développés dans l'équipe de Dorothée Missé.

Liste des publications

1. Imipramine Inhibits Chikungunya Virus Replication in Human Skin Fibroblasts through Interference with Intracellular Cholesterol Trafficking

   Sineewanlaya Wichit, *Rodolphe Hamel, Eric Bernard, Loïc Talignani, Fodé Diop, Pauline Ferraris, Florian Liegeois, Peeraya Ekchariyawat, Natthanej Luplertlop, Pornapat Surasombatpattana, Frédéric Thomas, Andres Merits, Valérie Choumet, Pierre Roques, Hans Yssel, Laurence Briant and Dorothée Missé*

   Sci Rep. 2017, Jun 9;7(1):3145. doi: 10.1038/s41598-017-03316-5

2. *Aedes Aegypti* Saliva Enhances Chikungunya Virus Replication in Human Skin Fibroblasts

   Sineewanlaya Wichit, *Fodé Diop, Rodolphe Hamel, Loïc Talignani, Pauline Ferraris, Florian Liegeois, Hans Yssel and Dorothée Missé*

   J Invest Dermatol. 2017, in Revision

3. SAMHD1 enhances Chikungunya and Zika virus replication in human skin fibroblasts

   Sineewanlaya Wichit, *Rodolphe Hamel, Andreas Zanzoni, Fodé Diop, Alexandra Cribier, Loïc Talignani, Abibatou Diack, Pauline Ferraris, Florian Liegeois, Natthanej Luplertlop, Andres Merits, Hans Yssel, Monsef Benkirane and Dorothée Missé*

   Manuscript in preparation

4. The effects of mosquito saliva on dengue virus infectivity in humans.

   Sineewanlaya Wichit, *Ferraris P, Choumet V, Missé D.*

   Curr Opin Virol. 2016, 21:139-145.

5. Zika virus causes supernumerary foci with centriolar proteins and impaired spindle positioning

   Benita Wolf, ***Sineewanlaya Wichit***[†]*, Fode´ Diop*[†]*, Pauline Ferraris*[†]*, Coralie Busso, Dorothée Missé and Pierre Gönczy*

   Open Biol. 2017 Jan; 7(1): 160231.

6. African and Asian Zika virus strains differentially induce early antiviral responses in primary human astrocytes

   Rodolphe Hamel, Pauline Ferraris , ***Sineewanlaya Wichit*** *, Fodé Diop , Loïc Talignani , Julien Pompon , Déborah Garcia , Florian Liégeois , Amadou A. Sall , Hans Yssel , Dorothée Missé*

   Infect Genet Evol. 2017, 49: 134–137.

7. Zika virus: epidemiology, clinical features and host-virus interactions.

   Hamel R, Liégeois F, ***Wichit S****, Pompon J, Diop F, Talignani L, Thomas F, Desprès P, Yssel H, Missé D.*

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8. Biology of Zika Virus Infection in Human Skin Cells.

   Hamel R, ***Wichit S***[#]*, Dejarnac O*[#]*, Ekchariyawat P, Neyret A, Luplertlop N, Perera-Lecoin M, Surasombatpattana P, Talignani L, Thomas F, Cao-Lormeau VM, Choumet V, Briant L, Desprès P, Amara A, Yssel H, Missé D.*

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9. Inflammasome signaling pathways exert antiviral effect against Chikungunya virus human dermal fibroblasts.

   Ekchariyawat P, Hamel R, Bernard E, ***Wichit S****, Surasombatpattana P, Talignani L,Thomas F, Choumet V, Yssel H, Desprès P, Briant L, Missé D.*

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APPENDICES: Awards

Le Meilleur poster

de la Journées de l'Infectiopôle Sud 2016

La médaille de bronze: La fondation Infectiopôle Sud

de la Journées de l'Infectiopôle Sud 2015

Le Meilleur poster

de la Journées de l'Infectiopôle Sud 2016

La médaille de bronze: La fondation Infectiopôle Sud

de la Journées de l'Infectiopôle Sud 2015