Chioma M. Okeoma *Editor*

Chikungunya Virus

Advances in Biology, Pathogenesis, and Treatment

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Preface

Chikungunya virus (CHIKV) is a mosquito-transmitted virus associated with increased morbidity and causes a debilitating chronic musculoskeletal disease in infected humans. The mosquito genus *Aedes* is responsible for CHIKV transmission. *Aedes* mosquitoes were previously restricted to the tropical and subtropical countries of the world, but increased globalization has resulted in worldwide spread of CHIKV-transmitting *Aedes* mosquitoes, including the species *Aedes aegypti* and *Aedes albopictus*. Despite precautions being taken to contain the spread of CHIKV, the number of cases of traveler-associated and locally transmitted CHIKV keep increasing in many countries. In some countries, including the United States, CHIKV infection is a nationally notifiable condition reportable to government health protection agencies, such as the US Centers for Disease Control and Prevention. Sadly, there are no vaccines or effective therapies for CHIKV infection, leaving infected people to rely on their immune systems to fight the disease. Since the re-emergence of CHIKV in 1941, exciting discoveries have been made in various aspects of CHIKV research. We have done our best to report the current state of knowledge of CHIKV; however, much work remains to be done to understand fully the fundamentals of CHIKV interaction with the environment, the vector, and the hosts.

Chikungunya Virus is the first book of its kind and consists of 12 chapters written by leading experts in the broad areas of CHIKV epidemiology, CHIKV biology, mechanisms of infection and pathogenesis, host response to infection, and clinical syndromes. These chapters are independent but interrelated. The chapter on clinical syndromes highlights the complexity of CHIKV infection in patients and the current approach to managing CHIKV disease in the absence of definitive therapy. Therefore, *Chikungunya Virus* will be of great interest to a wide audience and is intended for researchers, educators, postdoctoral and medical fellows, graduate and undergraduate students, health practitioners, and other public health officials.

Iowa City, IA, USA Chioma M. Okeoma

Contents

Clinical Syndrome and Therapy 1
Sylvie Abel and André Cabié

Host Response and Mechanisms of Subversion of Chikungunya 19
Lisa F.P. Ng and Pierre Roques

Epidemiological History of Chikungunya Virus 33
Ann M. Powers

Vaccines Against Chikungunya Virus Infection 45
Karl Ljungberg, Beate M. Kümmerer, Pierre Roques, Mariano Esteban, Andres Merits, and Peter Liljeström

Function of Chikungunya Virus Structural Proteins 63
Stefan W. Metz and Gorben P. Pijlman

Functions of Chikungunya Virus Nonstructural Proteins 75
Tero Ahola and Andres Merits

Interaction of Chikungunya Virus with the Mosquito Vector 99
Konstantin Tsetsarkin, Lark L. Coffey, and Scott C. Weaver

Chikungunya Virus Entry and Replication 127
Nathalie Chazal and Laurence Briant

Chikungunya Virus-Induced Autophagy and Apoptosis 149
Delphine Judith, Thérèse Couderc, and Marc Lecuit

Chikungunya Virus Pathogenesis 161
David W. Hawman and Thomas E. Morrison

Restriction Factors and Chikungunya Virus 185
Wadie D. Mahauad-Fernandez and Chioma M. Okeoma

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Clinical Syndrome and Therapy

Sylvie Abel and André Cabié

Introduction

Chikungunya virus (CHIKV), "that which bends up" in the Makonde dialect is an emerging alphavirus transmitted to humans by *Aedes* mosquitoes (*Ae. aegypti*, *Ae. albopictus*). CHIKV emergence in all the tropical zones at the beginning of the twenty-first century allowed experts to discover the polymorphism of its clinical manifestations (Weaver and Lecuit 2015).

The female mosquito becomes infected after feeding on blood of a viremic person (viremia for 5–7 days after onset of clinical signs). The virus replicates in the mosquito for a few days, and then the mosquito can transmit the virus to another person, throughout its life (Fig. 1; Schwartz and Albert 2010). Mother-to-child transmission can occur during childbirth when the mother is viremic (Gérardin et al. 2008). Although direct person-to-person transmission has not been reported, nosocomial transmission most probably occurs following blood transfusion or needlestick injury (Gallian et al. 2014; Parola et al. 2006).

CHIKV infection is most often symptomatic (≈80 % of cases); the symptoms last from a few days to several years depending on the case. In France, experts have defined three clinical stages (Simon et al. 2015): acute stage (from the first day on which the first symptoms appear (D1) up to day 21 (D21)); post-acute stage (from D21 to the end of the third month); and chronic stage (after 3 months; Fig. 1). This time staging takes into account the pathogenic, clinical, and therapeutic variations over time. The post-acute stage and a fortiori the chronic stage are not observed in all patients. The mortality rate of CHIKV is comparable to that of seasonal influenza (≈0.01 to 0.1 %), and is mainly related to the patient's age (increased over 75 years) and/or to chronic comorbidities (Schwartz and Albert 2010).

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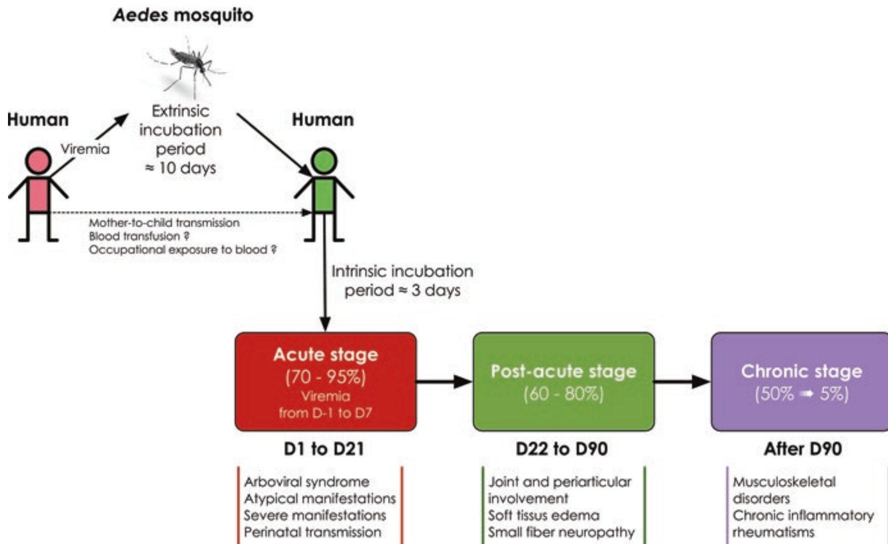

Fig. 1 Transmission cycle and natural history of chikungunya. D1 defined as the day of symptom onset

Acute Stage

Clinical Manifestations and Symptoms

Arboviral Syndrome

In contrast to other arboviral diseases, CHIKV infection is symptomatic in more than 70 % of cases, and ranges from 72 to 96 % (Appassakij et al. 2013). In symptomatic patients the mean incubation period is 3 days, range 1–12 days (Burt et al. 2012). In the common presentation, chikungunya is a rapid-onset febrile disease with no prodromal phase (Thiberville et al. 2013). High-grade fever occurs suddenly, along with inflammatory arthralgia and arthritis with periarticular edema and sometimes severe pain (Fig. 2). Joint pain is mostly polyarticular, bilateral, symmetrical, and occurs mainly in peripheral joints (wrists, ankles, and phalanges) and some large joints (shoulders, elbows, and knees). Sometimes arthralgia appears a few hours before the onset of fever. Other typical symptoms are: myalgia, headache, backache, macular to maculopapular rash, frequently associated with cutaneous pruritus (palms and soles) and edema of the face, lymphadenopathy. The rash appears after fever onset and is typically maculopapular involving the trunk and extremities but can also involve palms, soles, and the face (Fig. 3). External ear redness has been observed, and this may reflect chondritis and is evocative of CHIKV infection (Fig. 4) (Javelle et al. 2014). Fever and cutaneous

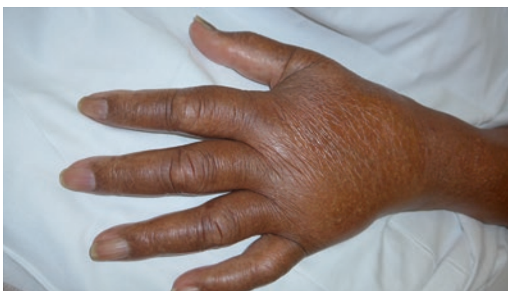

Fig. 2 Acute chikungunya: arthritis and distal edema

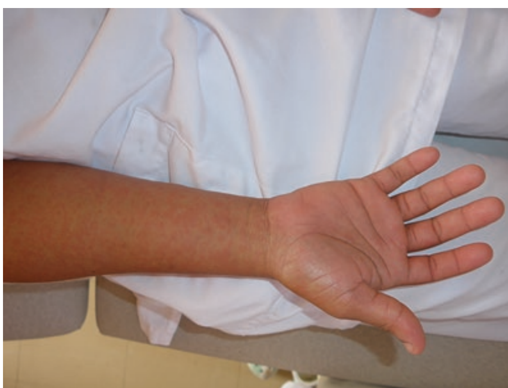

Fig. 3 Acute chikungunya: maculopapular rash and palm erythema

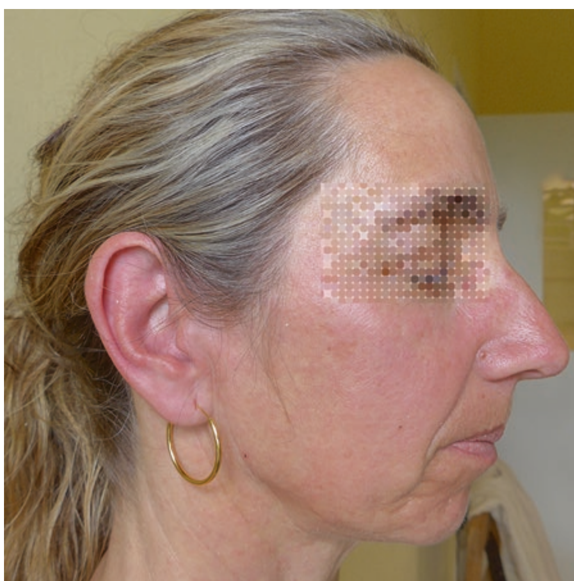

Fig. 4 Acute chikungunya: rash of the face and external ear redness

Table 1 Comparison of clinical and biological manifestations of dengue, chikungunya, and Zika virus infection

	Chikungunya	Dengue	Zika virus infection
Fever	+++	+++	++
Myalgia	+	+++	++
Arthralgia	+++	++	++
Retro-orbital pain	+	+++	+
Skin rash	+++	++	+++
Nonpurulent conjunctivitis	+	0	+++
Arthritis/edema	+++	0	++
Hypotension	+	+++	+
Minor bleeding	±	++	±
Lymphadenopathy	++	++	+
Thrombocytopenia	+	+++	±
Lymphopenia	+++	+++	±

rash last 3–5 days (viremic period), but articular manifestations may last 2–3 weeks in some patients (Thiberville et al. 2013). Asthenia and anorexia are common after regression of fever. The main laboratory finding is lymphopenia, which is closely associated with viremia when the lymphocyte count is less than 1000 per cubic millimeter. Other laboratory abnormalities include moderate thrombocytopenia, increased levels of aspartate aminotransferase, alanine aminotransferase, and creatine phosphokinase, and mild increase in C-reactive protein levels (about 50–60 mg/L). The clinical presentation is similar to that of other arboviruses (dengue, Zika virus infection) and may pose diagnostic difficulties (Table 1; Ioos et al. 2014; Simon et al. 2011; Staples et al. 2009).

Atypical Manifestations

Atypical presentations are observed in 0.5 % of cases, mainly in vulnerable patients (young children, elderly patients, chronic alcohol abusers, patients presenting with chronic medical conditions including systemic lupus; Economopoulou et al. 2009; Rajapakse et al. 2010). Atypical presentations include hyperalgic symptoms, gastrointestinal symptoms (diarrhea, vomiting, abdominal pain), and neurological symptoms (confusion, optic neuritis), damage to mucous membranes (oral or genital ulceration, conjunctivitis), and malaise (hypotension, dysautonomia). CHIKV can directly induce severe atypical presentations (rhabdomyolysis, bullous dermatosis, fulminant hepatitis, encephalitis or encephalopathy, Guillain-Barré syndrome, polyneuropathy, myocarditis, multiple organ failure; Betancur et al. 2016; Das et al. 2010; Farnon et al. 2008; Lemant et al. 2008; Simon et al. 2008). More frequently, it causes decompensation of chronic cardiac, respiratory, renal, systemic (lupus), and metabolic (diabetes) diseases, or various complications (dehydration, thromboembolism, loss of autonomy). The risk of drug toxicity by overdose

(self-medication) or drug interaction is high for acetaminophen as well as for other analgesics, anti-inflammatory drugs, long-term treatments, and traditional medicines used for self-medication.

Neonatal Chikungunya

CHIKV infection has not been linked to increased risk of miscarriage, fetal death in utero, or birth defects. However maternal–neonatal transmission can occur in viremic women during childbirth. Fifty percent of neonates are infected when they are born the day before or 5 days after the mother's first symptoms (Ramful et al. 2007). Neonatal chikungunya can either be congenital or neonatal (by mosquito bite after birth). Infected neonates exhibit atypical clinical presentation (fever, difficulty to breast-feed, and pain) occurring after a median incubation period of 4 days (3–7 days; Gérardin et al. 2008; Ramful et al. 2007). The main laboratory findings are thrombocytopenia, lymphopenia, and moderate increased levels of aspartate aminotransferase and alanine aminotransferase in blood. Severe manifestations occur in 50 % of cases, which include encephalopathy with progressive cerebral edema, hemodynamic disorders inducing severe sepsis, hemorrhagic complications due to intravascular coagulation, and cardiomyopathy. The mortality rate of severe presentations is 50 % and the risk of post-encephalopathy psychomotor sequelae is important (Gérardin et al. 2014).

The disease presentation in infants and children is often similar to that of adults. Nevertheless, some atypical or complicated presentations have been reported including hyperalgesia resistant to analgesic treatment, extensive bullous rash, hemodynamic disorders, dehydration, food intolerance, seizures, and meningeal syndrome.

Clinical Assessment

The first step is to discuss CHIKV infection in a patient with an acute onset presentation. A suspected case of acute chikungunya is defined by the combination of fever >38.5 °C and sudden onset of debilitating joint pain without any infectious focus. However, less symptomatic or atypical presentations have been reported. Physicians should be aware of other possible diagnoses in tropical areas: dengue, Zika virus infections, meningitis, malaria, and leptospirosis. The absence of joint involvement, predominance of myalgia, a hypotensive or bleeding trend, abdominal pain, and fever for more than 5 days may justify search for other diagnoses.

The clinical step allows identifying proven clinical signs of severity, atypical and/or complicated presentations (intense pain, organ failure, bleeding, dehydration, decompensation of comorbidity, thrombosis), pregnant women, patients at risk of severe presentations (neonates, children with a history of febrile convulsion, elderly patients, chronic disease treatment, social isolation), and guiding the patient triage (hospital admission or consultation, outpatient management; Fig. 5).

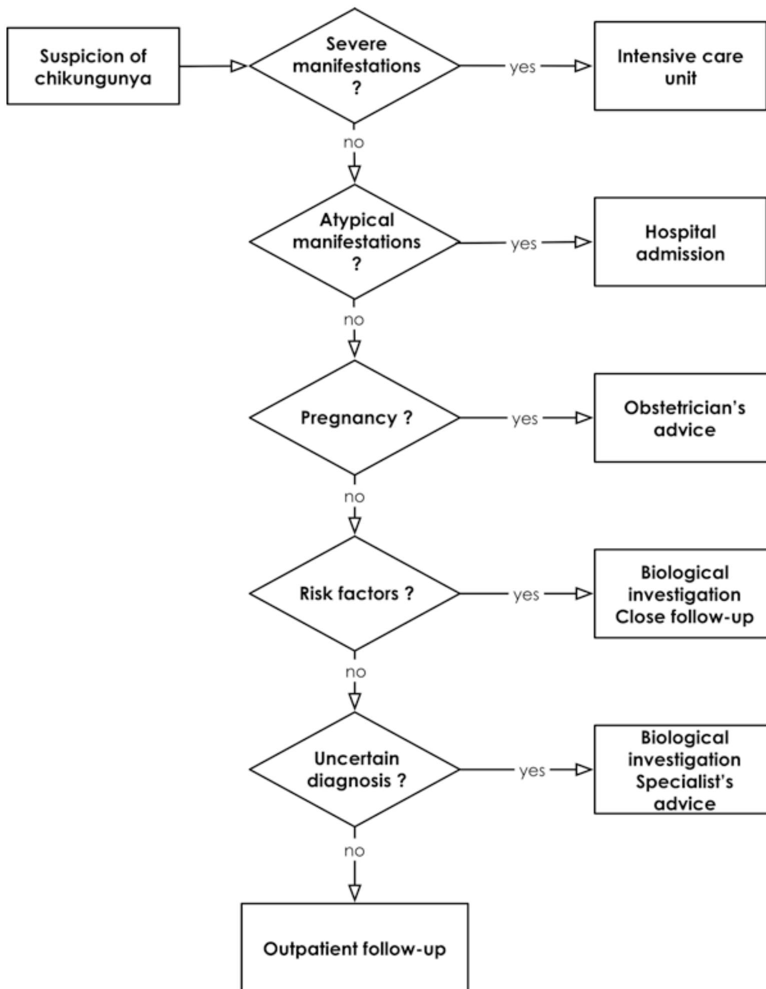

Fig. 5 Acute chikungunya: management of a suspected acute chikungunya

Some pediatric presentations may be atypical or complicated (hyperalgesia despite analgesic treatment, extensive bullous rash, hemodynamic disorders, dehydration, food intolerance, seizures, and meningeal syndrome) and should be referred to the emergency unit (Economopoulou et al. 2009; Ramful et al. 2007).

The suspicion of CHIKV infection in pregnant patients requires screening for signs of severity including fever >39 °C, neurological disorders, bleeding, uterine contractions, inability to eat, poor global health status, and/or alteration of the fetal heart rate pattern (FHRP) after 28 weeks. Any sign of severity requires emergency hospitalization, at best in obstetrics and neonatal resuscitation. A hospital consultation is recommended for any case of suspected chikungunya in the last 3 months of pregnancy. CHIKV infection suspected on the basis of a common presentation should be confirmed by ruling out other causes of potentially severe fever, according to the

clinical presentation, e.g., listeria, pyelonephritis, toxoplasmosis, rubella, malaria, dengue, Zika virus infection, and by recording the FHRP in the case of contractions, in order to define an obstetrical strategy. Furthermore, during epidemics, all patients in labor should be questioned about symptoms in the delivery room to identify any risk of CHIKV transmission for the unborn child.

Social isolation should also be taken into account to organize care, because of the great risk of rapid loss of autonomy among the weakest patients.

The clinical evaluation, in the acute stage, is sufficient to assess the impact of musculoskeletal lesions by identifying the site and the intensity of inflammatory manifestations. There is no indication for X-rays or ultrasound of the joints at this stage, except for another diagnosis.

Diagnosis of Acute CHIKV Infection

In contrast to dengue, routine biological tests are not essential for typical uncomplicated presentations in patients without any chronic disease or risk. The assessment of complete blood count, kidney and liver function, blood glucose, fluid and electrolyte level, and level of inflammation should be decided on a case-by-case basis. Screening for a differential diagnosis may justify implementing additional laboratory tests in the case of atypical clinical presentation, and complicated or abnormal outcome. Main differential diagnoses are dengue or Zika virus infection (possible coinfection), acute HIV infection, malaria, leptospirosis, sepsis, post-streptococcal immune reactions, and other acute viral infections.

The need for virological CHIKV infection confirmation depends on clinical manifestations and on the epidemic context. CHIKV infection confirmation is needed in cases of atypical or severe manifestations, in patients at risk of severe presentations (chronic diseases, extreme ages, pregnancy), in sporadic suspected cases, or in the first weeks of CHIKV emergence in a naïve population, as well as for other public health issues (study of strains, suspicion of a new focus, suspected post-epidemic cases). Conversely, in epidemic regions, diagnostic confirmation in the acute stage is not recommended during epidemics for typical cases without risk of severe presentation.

The confirmed diagnosis relies on virus detection through reverse transcriptase–polymerase chain reaction (RT-PCR) testing during the first week (Fig. 6; Simon et al. 2011). Specific antibodies detection is facilitated by the limited antigenic diversity of CHIKV and extensive cross-reactivity of the antibodies induced by different strains. Serum IgM is detectable from day 5 (and even earlier) to several months after the onset of illness and is also considered as a diagnostic parameter. Seroconversion can also be detected by an increase in IgG by a factor or 4 or more between the acute phase and convalescent phases. The tests are usually performed as follows: RT-PCR between Day 1 and Day 5, RT-PCR and serology between Day 5 and Day 7, serology alone after Day 7; viral culture is not routinely performed. The interpretation of the tests is based on the epidemiological context and clinical information provided by the clinician (time of onset of symptoms is mandatory).

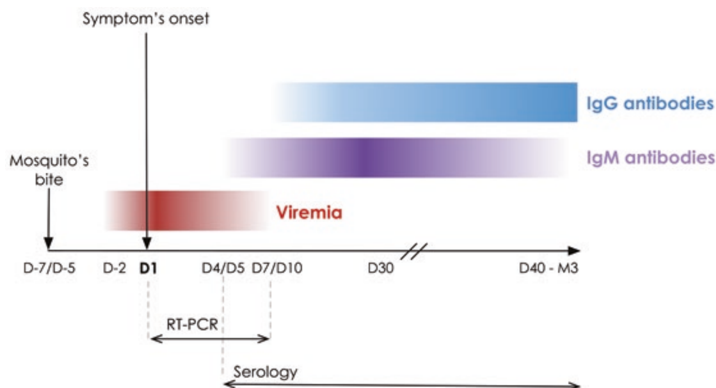

Fig. 6 Biological diagnosis of chikungunya

Therapeutic Management

General Principles

Thus far, no specific therapeutic agent is available for the treatment of infected persons. Treatment is symptomatic and should be adapted to the clinical context and medical status (risk groups). The purpose of treatment is to control fever, pain, dehydration, and to prevent organ failure, iatrogenic risk, and functional impairment. Preventing viral spread to relatives completes the management plan for CHIKV.

The analgesic treatment is based on acetaminophen (stage 1) in first intention. The risk of hepatitis, sometimes fulminant, is increased in the acute stage of chikungunya by the conjunction of viremia and supra-therapeutic doses (maximum dose in healthy adult 60 mg/kg/day and not more to 4 g/day), of interactions (drugs, alcohol, traditional medicines), and comorbidities (liver disease, malnutrition, etc.). The use of nonsteroidal anti-inflammatory drugs (NSAIDs) and salicylates is not recommended within the 14 days after onset of the disease because of the risk of bleeding complications related to a possible dengue fever and Reye's syndrome induced by aspirin. The use of stage 2 analgesics (weak opioids) is required if acetaminophen is not effective, either as tramadol alone or in combination with acetaminophen. Morphine administered per os or subcutaneously should be discussed case by case, usually at the hospital and after a strict assessment of the risk–benefit ratio because of possible respiratory, digestive, neurological, and urinary complications.

Prescribing corticosteroids is not recommended, regardless of the route of administration, because it brings no benefit in the medium to long term, and because it promotes a severe rebound of arthritis and tenosynovitis (Fig. 7). This treatment should be discussed by specialists in the case of encephalopathy or neuritis.

It is recommended to prevent dehydration in every case (oral or parenteral fluid intake, stopping diuretics, etc.). The management of CHIKV infection includes screening for new chikungunya-related pathological events (pyrexia, bullous skin lesions, and organ involvement) and increased monitoring of cardiac, hepatic, renal,

Fig. 7 Post-acute chikungunya: severe rebound of arthritis (*black arrow*) and tenosynovitis (*white arrow*) after corticosteroid discontinuation

metabolic, and systemic comorbidities. Iatrogenic risk prevention should also be implemented by monitoring long-term treatments (including antihypertensive drugs), by complying with the prescribed maximum doses and combinations to be avoided, as well as by informing the patient about the dangers of self-medication (interactions, toxicity), including herbal medicines.

Physical measures complete the management, which include sick leave or any occupational adjustment to prevent exhaustion and hypersolicitation of inflammatory joints, removing rings and other tourniquet-acting devices in the case of edema, icing and/or partial immobilization in the case of arthritis (night orthosis), and prevention of decubitus-related complications as appropriate. Prescribing physiotherapy (active–passive mobilization) should be discussed in the case of adverse outcome after 1 week if there is a risk of functional loss, as well as analgesic physiotherapy for pain resistant to analgesics. Social care may be required (housekeeper, frequent visits of a nurse or close relatives) for fragile patients at risk of aggravating or losing their autonomy.

Severe Manifestations

Severe manifestations must be managed in a hospital with an appropriate intensive care unit. Using immunoglobulins is indicated in cases of chikungunya-related Guillain-Barré syndrome.

Pregnant Women

The recommended symptomatic treatment is acetaminophen, with a maximum dose of 1 g × 4/day. All NSAIDs (including aspirin and topical presentations) are contraindicated after 24 weeks of amenorrhea (risk of fetal renal failure and closure of the ductus arteriosus, eventually leading to fetal death in utero). The mother and relatives should be informed about the risks of self-medication and aromatherapy (hepatic enzyme induction).

The advice of an obstetrics specialist is required for the diagnosis when a woman is infected at the end of her pregnancy in order to assess the impact on the unborn child as well as for a possible obstetrical decision. Cesarean section has no proven protection against CHIKV transmission to the child (Ramful et al. 2007). Cesarean section is indicated in case of FHRP alteration, as with any threatening fetal distress. Effective tocolysis can delay delivery beyond the viremic phase, and decrease the risk of neonatal transmission. The ongoing CHIKIVIG-01 clinical trial in the French Caribbean territories and in French Guyana aims to evaluate the safety and effectiveness of intravenous hyperimmune anti-CHIKV immunoglobulins to prevent neonatal CHIKV infection in neonates of viremic mothers (No. ClinicalTrial.gov NCT02230163).

Neonates and Children

Sustained 7-day monitoring of neonates is implemented when the mother delivers and is suspected to be infected. If the mother is confirmed to be infected, and the neonate is born with an undetectable viral load, he or she must be monitored for at least 5 days in the maternity unit. Clinical surveillance includes body temperature, quality of breast-feeding, pain, skin condition (rash, edema of the extremities), and hydration level. The typical pediatric presentations are treated symptomatically as for adults.

Prevention

Applying individual antivectorial protection measures (mosquito nets, repellents adapted to the patient, air conditioning) is recommended for suspected cases of chikungunya in the acute stage in areas with *Aedes* circulation. This practice in addition to actions implemented to eradicate mosquito breeding sites will help to break the chain of transmission. CHIKV infection may be acquired by accidental exposure to the blood of a viremic patient. Standard precautions are recommended for prevention.

Post-Acute Stage (From D22 to D90)

The post-acute stage of CHIKV infection is mainly characterized by persistent joint pain in about 60 % of patients. Higher incidence is observed after 40 years of age and in female patients (Simon et al. 2011). The other parameters associated with

persistent joint symptoms are mainly: severity of the acute stage (high-grade fever, arthritis ≥6 joints, depression, high level of viremia), lack of rest in the acute stage, and previous musculoskeletal comorbidities.

Clinical Symptoms

The main characteristic of the post-acute stage is the persistence or the occurrence of multiple and polymorphic manifestations dominated by inflammatory manifestations: inflammatory arthralgia, arthritis (synovitis with or without effusion), tenosynovitis, bursitis, tendinitis with risk for tendon rupture, enthesitis, bursitis capsulitis, or periostitis. The trend is a continuous mode or inflammatory attacks frequently promoted by cold and associated with decompensation of pre-existing degenerative or traumatic arthropathy, and local events such as edema of extremities, tunnel syndromes, joint stiffness, or neuropathic pain (Fig. 8). The absence of anti-inflammatory treatment, untimely excessive physical stress, and even a complete and prolonged joint rest, can have a deleterious effect on clinical recovery. This post-acute stage may also include severe asthenia and neuropsychological disorders, particularly if pain is not controlled.

Clinical Assessment

An accurate semiotic analysis allows defining the diagnostic workup that determines the optimization of treatment. It should particularly discriminate between pain and functional impairment due to a persistent inflammatory process, and

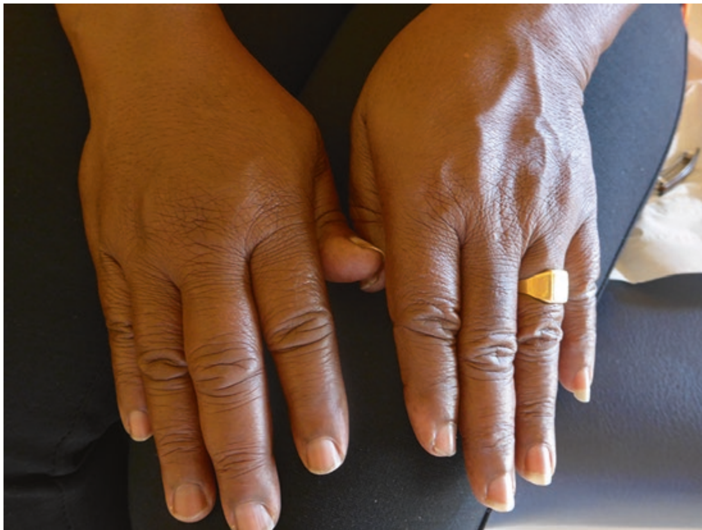

Fig. 8 Post-acute chikungunya: edema of extremities

symptoms related to decompensation of joints already altered by osteoarthritis or other processes. Indeed, treatment choices and effectiveness depend on the accurate assessment of lesions.

Biological Tests and Imaging

At this stage, it is essential to confirm serologically the diagnosis of CHIKV infection. Other laboratory tests are used to determine the level of inflammation and, as appropriate, to carry out a pretherapeutic assessment and screen for sources of comorbidities, such as rheumatic disease.

Imaging is not systematically performed at this stage, unless in case of diagnostic doubt or a severe disease lasting more than 6 weeks, as it may modify therapeutic choices (suspected pre-existing arthritis, tendon rupture, etc.). Plain radiographs and ultrasound of symptomatic joints should be used when clinical examination is not definitive enough. Consultation with rheumatologists is required in the case of inflammatory disease with painful and debilitating arthritis persisting beyond 6 weeks or if bone erosion is observed.

Therapeutic Management

The objective of treatment is to relieve the patient of pain and inflammation and to limit the consequences of the inflammatory process: joint stiffness, loss of muscle tone, and loss of physical fitness. The treatment is implemented by the general practitioner (GP) who takes into account the patient's clinical presentation, comorbidities, and socioeconomic status.

The therapeutic approach is primarily based on analgesics (stage 1 and 2, antineuropathic drugs) and NSAIDs. Analgesia should be optimized by combining a stage 1 or 2 analgesic agent, depending on the pain, with an agent targeting the painful neuropathic component (e.g., nefopam, pregabalin, gabapentin) if necessary, and active physical therapy on the persistently painful areas. Stage 3 agents may be used when stage 2 analgesics combined with an appropriate anti-inflammatory treatment have failed. Consulting a pain specialist is advised. No NSAID class has demonstrated superiority of effectiveness on post-acute chikungunya symptoms. This treatment is prescribed at full dose unless contraindicated, in taking care to cover the night by taking an evening and/or extended-release formulation. The effectiveness of NSAIDs should be reassessed (dose, schedule) during the first week; an inadequate response on the 10th day imposes a switch to another class of NSAIDs. It is important to continue NSAID treatment for several weeks; if well tolerated, gradually wean the patient.

Systemic corticosteroids should only be used for highly inflammatory polyarticular presentations, especially when associated with tenosynovitis, active synovitis, or in the case of resistance or contraindication to NSAIDs. The dose of 10 mg/day of prednisone for 5 days with de-escalation within 10 days is usually sufficient for refractory to moderate NSAIDs. A 0.5 mg/kg/day dose of prednisone for 5 days, with gradual weaning for 10 days is used for the most severe presentations.

A local anti-inflammatory therapy (topical or infiltration) should be prescribed in the case of tenosynovitis, bursitis, tunnel syndrome, capsulitis, or synovitis, so as to limit the therapeutic excess. However, the surgical decompression of a tunnel syndrome is not advised in an inflammatory context because of the risk of poor healing and reflex sympathetic dystrophy syndrome.

There is no indication to initiate disease-modifying antirheumatic drug (DMARD) therapy before 8 weeks in the post-acute stage, with a specific antirheumatic agent such as methotrexate. This treatment may be initiated only in patients with persistent arthritis, after screening for inflammatory arthritis and consultation with a rheumatologist. The effectiveness of hydroxychloroquine has not been established in this indication.

The benefit of physical medicine depends on lesion assessment and the disease's overall impact (pain, autonomy, quality of life). The expected benefits are pain relief, preserving the range of motion, and muscle tone.

Assessing the psychological and social impact of the disease completes the management of patients with persistent symptoms, which supports the relevance of a regular assessment of pain. Using psychotropic treatments, occupational adaptation, or requiring social assistance is decided on a case-by-case basis.

Chronic Stage (After D90)

In CHIKV infection, the chronic stage is defined by the absence of return to the preexisting condition more than 3 months after the onset of CHIKV infection. The chronic phase can last a few months to several years. Schematically, the disease progresses to cure without sequelae, spontaneously or after treatment, or to a prolonged persistence of joint and/or general symptoms, or to aggravation because of an inflammatory or degenerative process (Borgherini et al. 2008; Bouquillard and Combe 2009; Burt et al. 2014; Gérardin et al. 2013; Sissoko et al. 2009). Impaired quality of life has been reported by most chronic patients in the years immediately following CHIKV infection. The proportion of chikungunya patients who fully recovered, partially improved, or had persistent symptoms vary between studies and according to the time of the study assessment from the onset of the disease. In all cases, the frequency of persons presenting with symptoms of chronic chikungunya decreased with increasing time of onset.

Clinical Symptoms

The observed clinical symptoms are the same as in the post-acute stage. An accurate serological analysis allows identifying the type of lesion site, as in the post-acute stage. The diagnostic approach consists of qualifying the nosology of each patient according to the presence or absence of inflammatory symptoms (arthritis, enthesitis, tenosynovitis, inflammatory arthralgia) and the number of joints involved (polyarticular if ≥4 joints). The level of clinical inflammatory activity and its functional impact should also be taken into account.

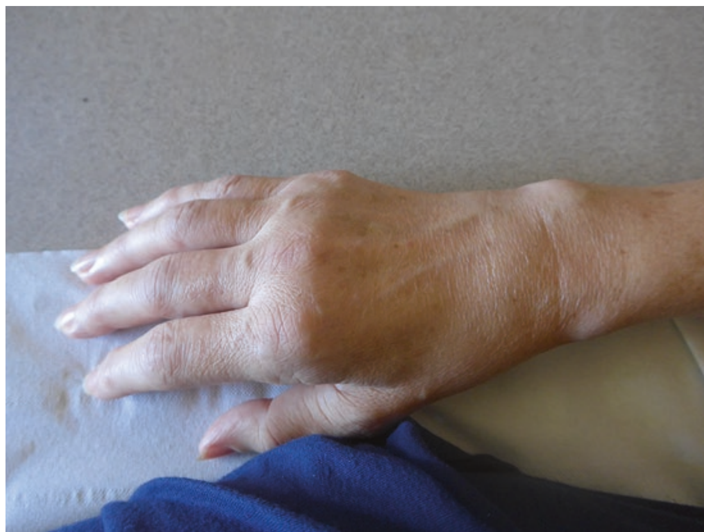

Fig. 9 Chronic chikungunya: rheumatoid arthritis 9 months after acute chikungunya virus infection

Chronic inflammatory rheumatisms (CIR; Fig. 9) are different from musculoskeletal disorders (MSDs; Fig. 10). The former has the most severe functional prognosis; the latter are by far the most frequent (95 %).

Rheumatoid arthritis (RA) is the most common post-chikungunya CIR before spondyloarthritis (SA). Otherwise, a nondestructive arthritis not meeting the criteria for RA or SA (Aletaha et al. 2010; Rudwaleit et al. 2011) is called undifferentiated polyarthritis (IP), regardless of age, and only after ruling out other causes of polyarthritis (microcrystalline, autoimmune, granulomatous secondary to chronic viral hepatitis, etc.). At the individual level, CIR depends on the absence of rheumatic signs before infection, the continuous symptoms of acute infection to CIR, CHIKV seropositivity, and ruling out of differential diagnoses. Patients not meeting the definition of post-chikungunya CIR are classified as presenting with other MSDs.

Biological Tests and Imaging

It is essential to confirm the diagnosis by CHIKV serology, as for the post-acute stage, if it has not been done before. Other laboratory tests can be performed in order to assess the level and compare the clinical, biological (especially rheumatoid factor, ACPA, ±HLA B27), and imaging data to the current criteria for RA and SA for all patients presenting with at least one chronic synovitis resistant to an appropriate treatment at the post-acute stage (>6 weeks).

Imaging may be prescribed (X-rays, ultrasound, MRI) according to clinical findings and the diagnostic hypothesis that follows.

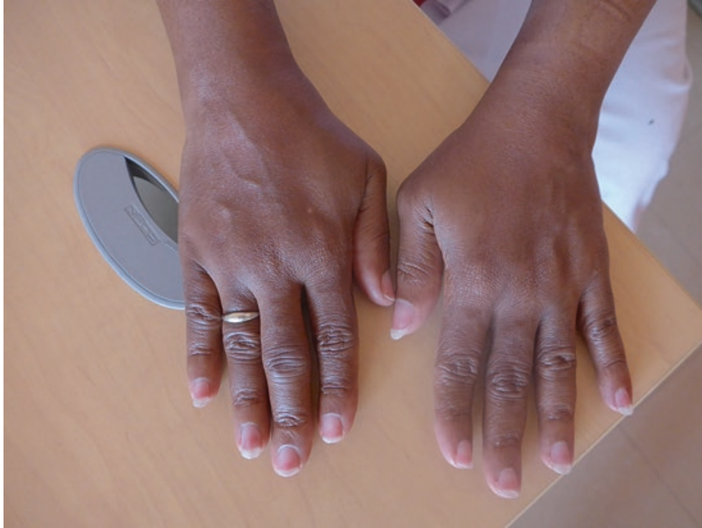

Fig. 10 Chronic chikungunya: musculoskeletal disorders (polyarthralgia, edema)

Thus, at the end of this diagnostic step, the practitioner may identify the type of persistent post-chikungunya symptoms and propose an individualized treatment based on the diagnosis, functional prognosis, and patient's condition.

Therapeutic Management of Chronic Inflammatory Rheumatisms

The management of post-chikungunya CIR, with or without joint destruction, systematically requires the advice of a rheumatologist, at best in the context of a multidisciplinary meeting. This management should begin during the first month of the chronic stage for better effectiveness (remission or cure). The treatment goals are to limit the potentially destructive outcome, to decrease the functional and psychosocial impact, and to improve the quality of life. Chikungunya-induced CIR can justify postponing a long-term treatment. This treatment must comply with international guidelines for RA and SA (Calabrese et al. 2016; Ward et al. 2016). The treatment modalities are well defined for both diseases; methotrexate has a special position in these guidelines.

Therapeutic Management of Other Musculoskeletal Disorders (Non-CIR)

The management of musculoskeletal disorders (MSD) persisting after 3 months is based on the same principles as the management of post-acute presentations, that is, preventing persistent inflammation and pain, and treating associated factors.

Treatment should always be optimized by combining an analgesic, a NSAID, a local anti-inflammatory therapy in resistant sites (including joint or peritendinous infiltration), and physical therapy (see above). The therapeutic effectiveness can be assessed in the medium term, for several weeks.

A short corticosteroid therapy (regimens similar to those proposed for the post-acute stage) must be sparingly used for multiple TMS not controlled by the first-line treatment, such as multiple hypertrophic tenosynovitis or distal edematous polyarthralgia. A switch with NSAIDs is recommended to limit the clinical rebound after weaning of the corticosteroid therapy. Consulting a rheumatologist and if necessary a physical medicine specialist, is recommended for all cases resistant to or dependent on corticosteroids (weaning not possible or relapse imposing successive courses). The consulted specialist may have to requalify the diagnosis when appropriate and should find a general or local therapeutic alternative (e.g., infiltration) to spare corticosteroid use, especially in patients at high risk of adverse effects (osteoporosis and other debilitating bone diseases, diabetes, hypertension, etc.).

The inadequate response to treatment including infiltration of an isolated arthritis requires a regular diagnostic reassessment so as not to overlook evolution to a CIR, and especially SA. As for CIR, the management also includes dealing with identified comorbidities, dealing with the psychological, social impact, and management of the musculoskeletal system.

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Host Response and Mechanisms of Subversion of Chikungunya

Lisa F.P. Ng and Pierre Roques

Chikungunya Fever and Disease Manifestation

Chikungunya fever (CHIKF) is an arthropod-borne viral disease transmitted by the *Aedes* mosquitoes, and is characterized by fever, headache, rashes, and debilitating arthralgia (Pialoux et al. 2007; Robinson 1955). Caused by the chikungunya virus (CHIKV), an alphavirus belonging to the *Togaviridae* family, the virus has an incubation period of 3–7 days (Powers and Logue 2007). Although only up to 15 % of asymptomatic cases were reported in patients, CHIKF remains primarily a nonfatal incapacitating disease. However, severe forms including deaths, often associated with comorbidities have been reported in the 2005–2006 Indian Ocean islands outbreaks (Lemant et al. 2008; Mavalankar et al. 2007). Similar clinical manifestations were also described from the new wave of CHIKV outbreaks in the French West Indies and Caribbean islands since November 2013 (Leparc-Goffart et al. 2014). The virus has since spread to several parts of Central and Latin America (Morens and Fauci 2014; Weaver and Lecuit 2015).

Typical disease symptoms in most patients (>85 %) include abrupt febrile illness (temperature usually >38.9 °C), maculopapular rash with articular pains. Other symptoms include myalgia, headache, edema of the extremities, ocular manifestations, and gastrointestinal symptoms (Borgherini et al. 2007; Lakshmi et al. 2008), and may be linked to direct or indirect effects of viral replication in these tissues

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(Ozden et al. 2007). Rheumatic manifestations in up to 50 % of the adult patients (6 months to 1 year PI) typically consisted of a febrile arthritis mainly affecting the extremities (ankles, wrists, phalanges; Borgherini et al. 2008; Brighton and Simson 1984; Fourie and Morrison 1979; Manimunda et al. 2010; Schilte et al. 2013; Simon et al. 2007; Sissoko et al. 2009).

CHIKF is usually benign in children. Atypical manifestations with subsequent sequelae have also been described in newborn babies such as neurological manifestations ranging from simple and complex febrile seizures to meningeal syndrome, acute encephalopathy, diplopia, aphasia, acute disseminated encephalomyelitis, and encephalitis (Le Bomin et al. 2008; Lewthwaite et al. 2009; Robin et al. 2008; Valamparampil et al. 2009). Severe skin blistering has also been described with intraepidermal vesiculobullous lesions (Robin et al. 2008; Valamparampil et al. 2009). Conversely, persistent arthralgia and exacerbation of underlying medical conditions are rare in children.

Notably, the epidemics in La Réunion were the first evidence with severe adult cases and deaths due to CHIKF (Economopoulou et al. 2009). These cases occurred on underlying medical conditions (cardiovascular, neurological, and respiratory disorders). Furthermore, there was a 22 % increase in adult patients with Guillain-Barré syndrome that required respiratory support during the La Réunion outbreak (Lebrun et al. 2009). This phenomenon was also observed in the 2014–2015 epidemic in French Polynesia (Lastère S unpublished). Taken together, atypical severe clinical manifestations as a result of CHIKV infection accounted for close to 1.5 % of the total infected population (4147 hospitalized out of 266,000 cases in La Réunion; Soumahoro et al. 2011; Renault et al. 2012). Fortunately, acute organ dysfunction comprised less than 0.2 % of the total severe cases (Renault et al. 2012; Cabié et al. 2015).

It is important to note that CHIKV also had profound acute arthritogenic activities in patients over 60 years of age that could have contributed to chronic incapacitating arthritis described in other alphaviral diseases in Australia, South America, and Northern Europe (Harley et al. 2001; Levine et al. 1994; Suhrbier and La Linn 2004; Tesh 1982; Toivanen 2008). Moreover, patients with post-CHIKV rheumatoid arthritis- (RA-) like illnesses were also reported (Chopra et al. 2008). The development of progressive erosive arthritis was also reported in some studies (Brighton and Simson 1984; Malvy et al. 2009; Manimunda et al. 2010). However, in contrast to what is known in canonical autoimmune RA, the levels of RF and anti-CCP antibodies were not elevated (Manimunda et al. 2010), thereby suggesting that post-CHIKV arthritis was a chronic inflammatory erosive arthritis. Nonetheless, the current lack of relevant animal models to study CHIKV-induced chronicity limits the understanding of these rare events.

Infection and Disease Pathogenesis: Human and Animal Models

Cutaneous manifestations that subsided without any sequelae in 3–4 days have been reported (Prashant et al. 2009). This eruption could be a hallmark of the inflammatory response of the skin (the portal of entry of the virus after the mosquito's bite)

that mobilized resident cells such as keratinocytes, melanocytes, and dermal fibroblasts (Couderc et al. 2008; Puiprom et al. 2013). CHIKV has been postulated to interact with resident dendritic cells (DCs) including Langerhans cells that contribute to virus spread to other target organs such as muscles, liver, kidney, heart, and brain (Kam et al. 2009).

In an effort to further understand the mechanisms of CHIKV pathogenesis, animal models have been established in mice and nonhuman primates. Studies on mouse models have been focused mainly on acute pathologies induced by CHIKV and disease severity. Notably, only some wild-type laboratory strains are susceptible to CHIKV infection (Ziegler et al. 2008; Gardner et al. 2010). Regardless of age and inoculation routes, susceptible mice in adult wild-type (Gardner et al. 2010; Teo et al. 2013), and also in newborn and young mice (Couderc et al. 2008; Ziegler et al. 2008; Morrison et al. 2011) develop viremia, and skeletal muscles exhibit severe necrotic myositis and high viral load. Pathological changes are also observed in joint-associated connective tissues adjacent to affected muscles. Although CHIKV RNA is cleared from most tissues within days after infection, viral RNA may persist in joint-associated tissues for at least 16 weeks, associated with histopathological evidence of joint inflammation (Hawman et al. 2013). In the case of severe disease, viremia is high and CHIKV also disseminates to other tissues, including skin and eye. In all these tissues, CHIKV-positive cells were identified as fibroblasts (Ziegler et al. 2008; Couderc et al. 2008). These findings are relevant for human disease, as similar tissue and cell tropisms have been observed in biopsy samples of CHIKV-infected human patients (Couderc et al. 2008, 2012). Together, these data demonstrate that infection of peripheral tissues associated with human CHIKV disease, joints, muscle, and skin, is mainly restricted to conjunctive tissues and that the fibroblast is a predominant target cell of CHIKV during acute CHIKV infection.

In the Cynomolgus macaque (*Macaca fascicularis*) model, both acute and chronic manifestations could be monitored. During acute infection, viremic levels up to 10^8 pfu/ml could be detected in CHIKV-infected macaques (Labadie et al. 2010; Roy et al. 2014; Messaoudi et al. 2013). At day 4 post-infection (pi) CHIKV could be detected in the cerebrospinal fluid of all tested macaques, but clinical neurological disease was detected only in macaques receiving the highest infectious doses (Labadie et al. 2010). Interestingly, the acute infection was tightly controlled given that the viral titer was reduced to basal levels at day 10 pi, similar to reports described in patients or mice (Ziegler et al. 2008). These viral replication profiles were also recorded in rhesus macaques (Akahata et al. 2010; Chen et al. 2010). Similar to patients, early leukopenia was observed (Akahata et al. 2010; Borgherini et al. 2007, 2009; Labadie et al. 2010) together with markers of IFN-α/β antiviral response, inflammation, and cell immune activation (Higgs and Ziegler 2010; Labadie et al. 2010; Messaoudi et al. 2013). Infection in pregnant rhesus macaques did not transmit the virus to the fetus in utero (Chen et al. 2010). Nonetheless, experimental infection of newborn macaques remains to be explored and these studies will be able to confirm the capacity of CHIKV to infect and replicate within immature brain tissues.

Cell Targets and Their Role in Pathogenesis

Both hematopoietic and nonhematopoietic cells have been demonstrated in the control of CHIKV infection by the innate immune system (Her et al. 2010; Schilte et al. 2010). Although nonhematopoietic fibroblasts have been reported to be susceptible to CHIKV replication (Sourisseau et al. 2007), it has been established that primary monocytes and macrophages are the major hematopoietic subsets targeted by CHIKV in virus-induced pathogenesis in both CHIKF patients and in animal models (Her et al. 2010; Hoarau et al. 2010; Labadie et al. 2010; Teng et al. 2012; Gardner et al. 2010). Furthermore, MCP-1 (Romano et al. 1997), a monocyte/macrophage chemoattractant (Lu et al. 1998) was shown to be significantly associated with the acute phase of CHIKV infection both in patients and animals (Chen et al. 2010, 2014; Gardner et al. 2010; Hoarau et al. 2010; Teng et al. 2015). In animal models, the high levels of MCP-1 were accompanied by increased infiltration of monocytes into the site of inflammation (Gardner et al. 2010; Labadie et al. 2010; Poo et al. 2014a), thus allowing newly produced viruses by the fibroblasts to infect monocytes/macrophages. To further support this hypothesis, treatment with MCP-1 inhibitor Bindarit (Bhatia et al. 2005) was demonstrated to abolish CHIKV-induced pathology completely (Rulli et al. 2011; Chen et al. 2014).

In macaques, CHIKV could persist in target tissues after its clearance from the blood, as demonstrated by immunohistochemistry and viral RNA detection using PCR and in situ hybridization assay (Labadie et al. 2010). At 7 or 9 days pi, CHIKV was detectable in nearly every organ or compartment tested: joints, secondary lymphoid organs, and, to a lesser extent, muscles up to 3 months pi. CHIKV was shown to replicate in several cell types during the acute phase (Higgs and Ziegler 2010; Labadie et al. 2010), but thereafter was detected mainly in macrophages by immunohistochemistry. CHIKV-infected monocytes and macrophages could be detected in the blood 6 h after infection (Roques et al. 2011) and in most tissues in the following day (by in situ hybridization, immunohistochemistry, RT-PCR, and virus isolation). Significant macrophage infiltration was also detected by histology throughout the study and long after virus clearance in blood (Labadie et al. 2010). Similarly, CHIKV was demonstrated to infect primary macrophages in vitro (Rinaldo et al. 1975; Sourisseau et al. 2007), resulting in the production of highly variable amounts of virus, from 10^3 to 10^6 pfu per ml (Gardner et al. 2010; Hoarau et al. 2010; Krejbich-Trotot et al. 2011a, b; Labadie et al. 2010; Sourisseau et al. 2007). However, CHIKV infection in $CCR2^{-/-}$ knockout mice resulted in a more severe, prolonged, and erosive arthritis, with no effect on virus replication (Poo et al. 2014a). Loss of CCR2, which is the receptor for MCP-1, caused a drastic change in the profile of infiltrating immune cells, coupled with a dysregulation of both pro- and anti-inflammatory pathways. Altogether, these data support the role of monocytes/macrophages as the cellular vehicle for virus dissemination, as well as a cellular reservoir for persistent CHIKV infection in immune-competent mammals.

Other than the increased infiltration of monocytes/macrophages, NK cells were also observed in large quantities in the inflamed joints of infected mice (Gardner et al. 2010). Furthermore, IL-12, which stimulates NK cell activity (Orange and Biron 1996),

was also present in high quantities, suggesting that activated NK cells play significant roles during CHIKV infection (Nakaya et al. 2012; Teo et al. 2015). Clinically, the role of these cells has been verified in natural CHIKV infection in humans where NK cells from CHIKF patients were strongly activated within the first days post-infection and led to a more sustainable CD4/CD8 response against several viral proteins (Hoarau et al. 2013; Petitdemange et al. 2011; Wauquier et al. 2011).

Separately, osteoblasts have been shown to be infected by CHIKV and drive osteoclatogenesis in vitro (Noret et al. 2012). This was confirmed by patient cohort studies where high levels of RANKL/osteoprotegerin (OPG) detected in CHIKV patients could be associated with macrophage-derived osteoclasts (Her et al. 2012; Chen et al. 2014a, b). Osteoclasts are known to cause bone erosion, indicating the importance of these cells in bone destruction in alphavirus-induced pathology (Noret et al. 2012; Phuklia et al. 2013; Chen et al. 2014a, b).

Innate Immune Response and Inflammation

Fever experienced by all CHIKF patients could be attributed to cytokines such as IL-1β, IL-6, and TNF-α, which are known pyretics (Ng et al. 2009). These cytokines have also been detected at high levels in acutely infected patients (Chow et al. 2011; Wauquier et al. 2011; Kelvin et al. 2011) and the levels returned to normal after fever and viremia have disappeared (Chow et al. 2011; Wauquier et al. 2010; Kelvin et al. 2011).

Arthralgia experienced by CHIKF patients closely resembles the symptoms induced by other alphaviruses (Pialoux et al. 2007; Powers and Logue 2007; Suhrbier and La Linn 2004). It is characterized by severe joint pain due to inflammation and tissue destruction caused by inflammatory cytokines such as IL-1β, IL-6, and TNF-α as reported in CHIKF patients (Ng et al. 2009; Hoarau et al. 2010; Chow et al. 2011). Prostaglandins have also been shown to be highly expressed by CHIKV-infected fibroblasts (Fitzpatrick and Stringfellow 1980) and may contribute to mechanisms of nociceptor activation and sensitization as described in osteoarthritis joints (Fitzpatrick and Stringfellow 1980; Malfait and Schnitzer 2013).

The specific involvement of cytokines and chemokines have shown IL-1β, IL-6, and RANTES to be associated with disease severity during the acute phase, thus enabling the identification of patients with poor prognosis and monitoring of the disease (Ng et al. 2009). Higher concentrations of pro-inflammatory factors such as IFN-α, IL6, and IP-10 were also found in patients with alphavirus-induced polyarthritis than in patients without, indicating a potential causative role in chronic joint and muscle pains (Hoarau et al. 2010; Ng et al. 2009; Wauquier et al. 2011). Different patient cohorts have reported different patterns of the inflammatory immune mediators, suggesting that the basal levels of these mediators differ in the different populations (Teng et al. 2015). Specifically, pro-inflammatory cytokines such as IL-6, MCP-1, and IFN-α were found to be elevated during the acute phase of the disease in several patient cohorts (Ng et al. 2009; Hoarau et al. 2010; Chow

et al. 2011; Kelvin et al. 2011; Wauquier et al. 2011). Positive correlation was also observed between the expression of IL-6 or MCP-1 and the high viral load in CHIKV-infected patients (Chow et al. 2011). Interestingly, IL-6 and GM-CSF were also observed to associate with persistent arthralgia (Hoarau et al. 2010; Chow et al. 2011). A meta-analysis comparative study demonstrated that pro-inflammatory cytokines such as IFN-α, IFN-β, IL-2, IL-2R, IL-6, IL-7, IL-12, IL-15, IL-17, and IL-18; anti-inflammatory cytokines such as IL-1Ra, IL-4, and IL-10; chemokines: granulocyte colony-stimulating factor (GM-CSF), IP-10, MCP-1, monokine induced by gamma interferon (MIG), macrophage inflammatory protein (MIP) 1α and MIP-1β; and growth factor: basic fibroblast growth factor (FGF) formed a generic acute CHIKV signature in all the different patient cohorts around the world (Teng et al. 2015). Although their respective roles are not fully understood, the various biomarkers indicated the important role that cytokines play in the pathology of CHIKV infection and can potentially lead to the development of modulators to reduce disease severity and halt disease progression.

The production of type I interferons, IFN-α and IFN-β, is the signature of an antiviral state in vertebrate hosts and they are essential to the functioning of the innate immunity against the replication and spread of virus. Type I IFNs and IFN-stimulated genes (ISGs) act through diverse mechanisms against viral invasions (Akira and Takeda 2004; Stetson and Medzhitov 2006). Although CHIKV was first reported to be a potent inducer of type I IFNs during infection as early as the 1960s (Gifford and Heller 1963), their roles in CHIKV infections are poorly known. Studies in patient cohorts have shed light on the interplay between type I IFNs and CHIKV during infections (Ng et al. 2009; Chow et al. 2011; Hoarau et al. 2010; Schilte et al. 2010), and experimental animal models have deciphered the role of RIG-I like receptors, Toll-like receptors, IRF 3/7, and interferon-stimulated genes (ISG15, Viperin, OAS) in limiting CHIKV replication (Brehin et al. 2009; Rudd et al. 2012; Schilte et al. 2012; Teng et al. 2012).

The role of type I IFN in CHIKV pathogenesis has been further investigated in human cells and mouse models. Data showed that infected nonhematopoietic cells sense viral RNA in a Cardif-dependent manner and participate in the control of infection through their production of type I IFN. Although the MAVS (also known as Cardif or IPS1) pathway contributes to the immune response both in cell culture of human fibroblasts and in mice, evidence for a MyD88-dependent sensor in preventing viral dissemination was demonstrated only in mice. It has been shown that interferon type I receptor (IFNAR) expression is required in nonhematopoietic cells but not in hematopoietic cells, as IFNAR$^{-/-}$ → WT bone marrow chimeras are able to clear the infection, whereas WT → IFNAR$^{-/-}$ chimeras succumb to disease. These data define an essential role for type I IFN, acting directly on nonhematopoietic cells, most likely fibroblasts, for the control of CHIKV (Schilte et al. 2010), although treatment with type I IFN is not a viable therapy when given after virus infection (Gardner et al. 2010). Other studies have also demonstrated that IRF3/IRF7-deficient mice developed hemorrhagic fever and shock after CHIKV infection (Rudd et al. 2012). Therefore, young age and inefficient type-I IFN signaling are risk factors for severe CHIKV disease.

Adaptive Immune Response and Protection

CHIKF leads to a protective adaptive immunity. The establishment of anti-CHIKV immune response after a primary infection could confer complete protection against reinfection. This provided the basis of the time-lapse between CHIKF epidemics (Laras et al. 2005). Anti-CHIKV IgM and IgG antibodies have been detected in the sera of infected patients during the acute phase of the infection (Panning et al. 2008; Kam et al. 2012a, b). The ability of anti-CHIKV antibodies to neutralize virus infectivity was also demonstrated by using sera from convalescent patients (Couderc et al. 2009; Kam et al. 2012a, b, c). These findings suggest that anti-CHIKV antibodies could be used as a potential prophylactic strategy against CHIKF (Couderc et al. 2009; Bréhin et al. 2008; Lee et al. 2011; Kam et al. 2012b, c; Pal et al. 2013; Smith et al. 2015). Therefore, viremic mothers and neonates born of viremic mothers, patients with severe neurological presentation of the disease, small infants, or adults with severe underlying comorbidities could benefit from passive immunization using anti-CHIKV immunoglobulins.

The importance of B cells was also demonstrated in B cell (μMT) knock-out mice infected with CHIKV, where viremia in these animals persisted for over a year, indicating a direct role for B cells in mediating CHIKV clearance (Lum et al. 2013). These animals exhibited a more severe disease than wild-type mice during the acute phase.

Antibody-mediated protection against CHIKV has been studied extensively for vaccine development (Ahola et al. 2015) and surface viral glycoproteins have been demonstrated to be key targets for protective neutralizing antibodies against CHIKV alphaviruses (Bréhin et al. 2008; Lee et al. 2011; Kam et al. 2012b, c; Pal et al. 2013). It was shown that immunization with CHIKV virus-like particle (VLP) vaccines and other vaccine candidates comprising key surface viral glycoproteins could induce the production of neutralizing antibodies and protect both mice and nonhuman primates against CHIKV challenge (Akahata et al. 2010; Kam et al. 2012b; Metz et al. 2013a, b; Hallengard et al. 2014; García-Arriaza et al. 2014; van den Doel et al. 2014; Roy et al. 2014). More recently, the first CHIKV VLP vaccine (Akahata et al. 2010) was successfully demonstrated to be well tolerated and protective in human trials, making it a significant breakthrough (Chang et al. 2014).

T cells are important effector cells during viral infection. Both $CD4^+$ and $CD8^+$ T cells can eliminate virus-infected cells. Adult $RAG2^{-/-}$, $CD4^{-/-}$, $CD8^{-/-}$, and wild-type C57BL/6 CHIKV-infected mice have demonstrated the importance of T cells in CHIKV-induced pathology (Teo et al. 2013). Interestingly, results indicated that CHIKV-specific $CD4^+$ but not $CD8^+$ T cells are essential for the development of joint swelling without any effect on virus replication and dissemination (Teo et al. 2013; Hawman et al. 2013). These observations strongly indicate that mechanisms of joint pathology induced by CHIKV in mice resemble those in humans, and differ from infections caused by other arthritogenic viruses such as Ross River virus (Morrison et al. 2006). Furthermore, using mice deficient for MHC II and IFN-γ, gene set enrichment analysis showed a significant overlap in differentially expressed genes from CHIKV arthritis and rheumatoid arthritis (Nakaya et al. 2012).

Challenges and Limitations to Fully Understand CHIKV Chronicity

To conclude, CHIKV infections induce a self-perpetuating pro-inflammatory reaction that causes arthralgia, explaining why pains are constant ailments in many patients with persistent joint-associated CHIKV even years after recovery from the initial febrile phase (Hoarau et al. 2010). No animal model could fully reproduce the chronic rheumatoid syndrome following CHIKF. Indeed, the disease pathology reported in mice is mainly driven by destruction of tissues with huge cell infiltration that could only be resolved 1–2 weeks after acute disease (Gardner et al. 2010; Morrison et al. 2011; Rulli et al. 2011). Despite virus persistence, severe joint damage is not always observed in macaques which could reflect the estimated scenario where only 5 % of patients meet the criteria for chronic inflammatory rheumatism (rheumatoid arthritis, spondyloarthritis, or unclassified polyarthritis; Chen et al. 2010; Labadie et al. 2010; Simon and Gasque 2015). Nevertheless, both animal models present inflammation, macrophage tissue tropism, and virus persistence in tissues (Labadie et al. 2010; Hawman et al. 2013). However, the exact mechanism in the establishment of chronic disease induced by CHIKV infection remains undefined.

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Epidemiological History of Chikungunya Virus

Ann M. Powers

Introduction

Chikungunya virus (CHIKV) is a mosquito-transmitted virus that has been known to cause human disease since 1952 when the virus was first characterized in an outbreak in East Africa. The virus became widely known on a global scale in 2013 when it entered the Western hemisphere and began to rapidly move through 45 counties in under 2 years. Although this expansion was highly visible and on a virtually unprecedented scale, CHIKV actually has a long history of both epidemic and low-level transmission throughout its endemic distribution in Africa and Southeast Asia. The historical epidemiological patterns of CHIKV are different from more recent activity. Apart from a few large outbreaks, activity in Africa has tended to involve only a few human cases at a time that are typically linked to close associations with the enzootic transmission cycle. In contrast, in Asia, where there is no known enzootic cycle, on-going, low-level activity is typical with large periodic urban outbreaks. The epidemiology of CHIKV since its re-emergence in 2004 in coastal Kenya has been characterized by large attack rates, rapid movement and geographic expansion, utilization of alternate mosquito vectors, and adaptation to novel ecologies. Understanding of the historical patterns of the virus as described below provides a framework for appreciating the modern movement of the virus.

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Discovery of CHIKV

In 1952–1953, the local populations of southern Tanganyika (current Tanzania) were affected by an epidemic of disease characterized by a fever with a rapid onset, joint pain, and rash. The pain was so debilitating and prolonged or recurring that the local word *chikungunya*, meaning "that which bends up" was given to the syndrome describing the stooped posture that resulted from the pain of the disease (Robinson 1955; Lumsden 1955). An alphavirus was later identified as the causative agent and given the name chikungunya virus (CHIKV) (Ross 1956). The outbreak occurred both in river valley lowlands as well as on the nearby Makonde Plateau where interestingly, the incidence was highest overall. It was speculated the incidence was highest on the plateau because no pre-existing antibodies were prevalent in this population as was the case in the valley areas. In contrast to the plateau areas, the lower valleys had mosquito populations that were continuously high (Jupp and McIntosh 1989) and the population was therefore likely more frequently exposed to CHIKV. However, distinct mosquito species may also have been involved in the contrasting affected ecologies which would have influenced the patterns of disease.

Approximately 150,000 people resided in the affected areas of Tanzania scattered among numerous small villages. Many of these villages on top of the plateau were far enough away from water sources that storage of fresh water was required (Lumsden 1955); this likely led to large populations of *Aedes aegypti* in close association with their homes promoting epidemic transmission as has been observed in recent epidemics (Chretien et al. 2007). A resulting overall morbidity rate of 47–50 % was reported among affected individuals on the plateau (Lumsden 1955). Although it has been speculated that there had been historical confusion between dengue and CHIKV outbreaks and that CHIKV outbreaks may actually have occurred as early as 1779 (Carey 1971), this first confirmed CHIKV epidemic in coastal Tanganyika demonstrated that this newly discovered virus was capable not only of significant human disease but also epidemics of arboviral disease at an unprecedented scale and rate.

Early Outbreaks and Periodic Detections

After the identification of CHIKV, a number of outbreaks occurred over the next 30 years that were attributed to this agent. These ranged geographically across central Africa from Senegal to Uganda south to South Africa, across the Indian Ocean to India and throughout Southeast Asia. However, although the outbreaks in Africa were more numerous, they tended to be small in scale whereas large urban outbreaks were primarily documented in Southeast Asia. Large urban outbreaks were first reported in Thailand in the late 1950s and early 1960s (Jupp and McIntosh 1989) and the scope of these outbreaks was staggering. For example, in Bangkok in 1962, up to 70,000 outpatient children were affected with CHIKV and the attack rate for the city was estimated to be 31 % (Halstead et al. 1969a). Clear evidence that the virus had been previously established in the area was seen in age-dependent

immune rates. Antibodies were found in 10–20 % of 1–2 year olds but 70–85 % of adults (>20 years) had CHIKV antibodies. Similarly high rates were found in rural Thailand (Halstead et al. 1969b) and in Vietnam after a 1964 outbreak (Deller and Russell 1968) further supporting the idea of long-term endemicity in the region. Curiously, after ~1982, CHIKV activity was virtually undetected in Bangkok even though most conditions for transmission remained present (Burke et al. 1985).

India also experienced large urban outbreaks of CHIKV in both the early 1960s to 1970s. A series of outbreaks was reported in Calcutta in 1963 and in Madras State from 1962 to 1964. The Madras activity involved an estimated 400,000 individuals in this single region in Southern India where the virus had not previously been documented (Myers and Carey 1967; Rao 1966). Even with an estimated 40 % of the population affected by CHIKV, the outbreak ended abruptly with activity nearly nonexistent by 1965 (Rao 1966). Interestingly, involvement of the central nervous system in children was identified in these outbreaks demonstrating early the ability of CHIKV to cause “atypical” disease (Jadhav et al. 1965; Carey et al. 1969) as had been reported in outbreaks since 2005. A later 1973 outbreak in central India (Barsi) also involved large attack rates and resulted in over 37 % of the population being infected (Padbidri and Gnaneswar 1979). Even though these outbreaks in India were significant, there was no reported epidemic activity from the country for the next 30 years. This curious apparent lack of CHIKV transmission does suggest the lack of establishment of an enzootic cycle in India after the cessation of epidemic activity.

During the 1950s to 1970s, a number of smaller scale outbreaks were recorded in Africa as well. Cases were reported in Zaire, Zambia, Senegal, Uganda, Zimbabwe, Nigeria, Angola, Central African Republic, and South Africa (Jupp and McIntosh 1988; Rodger 1961; Macrae et al. 1971; McIntosh et al. 1963, 1977; Moore et al. 1974; Tomori et al. 1975; Filipe and Pinto 1973). In some of these outbreaks, cases were reported in multiple areas of the country yet they had low attack rates in individual locations. This pattern suggested only low levels of transmission from the local vectors (McCrae et al. 1971). When low levels of activity were reported, it was postulated that the infections were a result of humans entering forest habitats when they were bitten by infected sylvatic vectors that tended to have a lower vectorial capacity than urban vectors due to a lower preference for human hosts compared with other vertebrates. These small outbreaks were periodic and covered a wide geographic range but rarely caused significant numbers of cases. This epidemiological pattern was quite distinct from that seen in Asia during the same time frame, perhaps most significantly due to the association of enzootic maintenance of CHIKV in Africa with a number of alternate vector species.

Re-Emergence of CHIKV

Perhaps not unexpected, due to the maintenance of CHIKV in zoonotic transmission in Africa, there was a re-emergence of epidemic CHIKV in 2004 in coastal Kenya. During July, an unusual increase in the number of malaria-like illnesses was detected in the island community of Lamu. However, local physicians noted that the

degree of joint pain associated with these cases was significantly more severe than that seen in standard malaria infections. In addition, 91 % of the blood smears were negative for parasites; thus, the increase in cases could not be accounted for by malaria. Additional testing for likely etiologies revealed positive CHIKV-specific antibodies and nucleic acid results (Sergon et al. 2008). The scope of this outbreak was quite large for eastern Africa with an estimated 13,500 cases. Yet recognition of the outbreak on a global scale was minimal.

Approximately eight months later, an outbreak of febrile illness began on the island of Comoros just off the coast of Tanzania. The outbreak was initially believed to be dengue but laboratory testing showed no evidence of dengue infections. Because of the recent CHIKV activity in Kenya, testing of samples for CHIKV was undertaken. Of 25 samples analyzed, 9 were positive for CHIKV antibody and 6 additional samples were RT-PCR positive. Similar to what was found in Lamu, a high percentage of patients reported fever and joint pain (>89 %) and a serosurvey performed during the outbreak revealed an attack rate of 63 % (Sergon et al. 2007). Molecular epidemiology further revealed that the virus originated from the Lamu/Mombasa outbreaks, demonstrating that the activity in Comoros was simply an extension of the Kenyan outbreaks rather than novel outbreaks (Kariuki Njenga et al. 2008). The total number of cases estimated in Comoros was nearly 215,000 resulting in a grand total of approximately 230,000 cases in just 1.5 years (Sergon et al. 2007). This outbreak also suggested the movement of epidemic activity rather than the cessation of a particular outbreak followed by periodic re-emergence of the virus elsewhere. This was a pattern that would characterize CHIKV outbreaks for the next decade.

Coincident with the large outbreak in Comoros was a smoldering outbreak in nearby La Réunion. Cases were first identified there in March of 2005 but the number of cases remained low until December with the onset of the rainy season (Bessaud et al. 2006). The peak of the outbreak occurred beginning the last week of January, 2006 when 45,000 cases were reported (Josseran et al. 2006) and an overall estimate of >244,000 cases was described (Renault et al. 2007). Intense curiosity regarding the reason for the very slow progression of this outbreak compared with the sweeping activity in Kenya and Comoros was addressed by microevolutionary analysis indicating a single amino acid change likely altered the mosquito infectivity of the strains that were isolated in 2006 (Schuffenecker et al. 2006). This mutation was postulated to enable the virus readily to infect the mosquito *Aedes albopictus*, which was far more abundant on the island than the traditional vector, *Aedes aegypti* (which was virtually absent from the island). The viral variant without this mutation was thought to be limited in ability to infect *Ae. albopictus*. This hypothesis was quickly confirmed using local mosquitoes and viral strains from early and late in the La Réunion outbreak (Vazeille et al. 2007) as well as infectious clones with engineered point mutations (Tsetsarkin et al. 2007). This was a significant finding demonstrating that a single mutation could affect the course of a global outbreak; had this mutation not emerged in the viral population late in 2005, the epidemic may have ended before the increase in cases in La Réunion and subsequent movement to India and Southeast Asia.

This Indian Ocean lineage of CHIKV continued its expansion by moving to India in 2006. After a 32-year absence of the virus, India reported an estimated one million cases in just one year in multiple areas of the country (Dash et al. 2007). From India, the virus was exported to a number of other countries in Southeast Asia including both endemic areas as well as locations with no previous documentation of transmission. Perhaps most notably, an exportation event from India to Italy resulted in the first autochthonous transmission in a subtropical area (Angelini et al. 2007). The activity in Italy was limited in both scope and duration, however, it further demonstrated the risk of transmission in areas where only *Ae. albopictus* were present as well as the ability of the virus to adapt to novel ecologies (Rezza et al. 2007).

Ongoing Threat of CHIKV from Endemic Areas

Although a viral mutation kept the Indian Ocean lineage outbreak alive, the threat of future CHIKV emergence from a different source was still present. Prior to and during the early re-emergence activity in 2004, CHIKV was continuing to circulate and cause large numbers of cases without substantial media attention. In particular, small yet substantial outbreaks were being reported in Central Africa and Indonesia. In 2000 in the Democratic Republic of Congo, an urban outbreak of CHIKV was detected after a 39-year absence of virus isolation in the country (Pastorino et al. 2004). An estimated 50,000 cases occurred in this outbreak with little awareness outside the area. Later, in 2006, CHIKV was identified as the causative agent in a number of febrile illness cases in Cameroon. The virus sequence obtained from this cluster revealed a high degree of homology with the strains from the Republic of Congo in 2000 suggesting continuous circulation of this lineage over at least 6 years in central Africa (Peyrefitte et al. 2007). Only about 400 cases of illness were reported during this outbreak, however, a follow-up cross-sectional serosurvey suggested that the recent infection rate was over 50 % (Demanou et al. 2010). Further evidence of transmission of this central African lineage was found in 2006–2007 in Gabon where a dengue-like outbreak occurred involving 20,000 suspected cases (Leroy et al. 2009). All this activity in central Africa was of the ECSA genotype, however, the lineage was distinct from that of the isolates associated with the Indian Ocean outbreaks (Peyrefitte et al. 2008). This outbreak in Gabon also linked transmission of the virus to the mosquito *Aedes albopictus* further signifying the importance of this species as an epidemic vector (Pages et al. 2009). The same virus lineage was also retrospectively linked to a cluster of febrile illness cases in children in Equatorial Guinea in 2002–2003 and again with travelers who visited this country in 2006 (Collao et al. 2010). Although samples from both time frames in Equatorial Guinea were of the same lineage, the 2006 samples were more closely related to samples more temporally similar from neighboring countries indicating continuous movement of the virus throughout this region over time. In 2011, a CHIKV outbreak affecting approximately 8000 individuals was reported in the Republic of Congo (Kelvin 2011). Genetic data from this outbreak were not

reported, but given the geographic location, it is reasonable that the continuously circulating central African lineage was progressing in both distribution and human infections with little global awareness.

Concurrent with this continuous transmission of the virus in central Africa was endemic transmission in Southeast Asia, particularly in Indonesia. Although antibodies against CHIKV were detected in Indonesia as early as 1972 (Kanamitsu et al. 1979), the virus was only first detected in Indonesia in 1982 when an outbreak was identified in South Sumatra. The epidemic quickly moved to numerous cities throughout Sumatra and a number of other islands of the archipelago were subsequently affected over the next 2 years including Java, Kalimantan, Bali, East Timor, East Nusa Tenggara, Papua, and Sulawesi. The attack rates ranged from 40 to 90 % depending upon the region (Porter et al. 2004). However, there were no additional reports of epidemic CHIKV illness in Indonesia for 15 years until a number of small outbreaks were reported between 1998 and 2003 on Java. Renewed activity was first reported in 1998–1999 in Yogyakarta, Java with a handful of clusters of febrile illness associated with arthralgia and rash. Interestingly, approximately 40 % of these cases exhibited either mild or asymptomatic infections. As activity increased, 24 distinct outbreaks were reported between 2001 and 2003 (Laras et al. 2005) moving across the country from northern Sumatra to Java to northeast Sulawesi and to Nusa Tenggara with the vast majority occurring on the main island of Java (83 %). Most of these epidemics lasted only 2–3 months and involved fewer than 200 individuals. The limited scope of each outbreak may have been due to the fact that most were in rural settings with only 21 % occurring in urban centers. The two most well- characterized outbreaks, in Bogor and Bekasi, showed repeated periods of peak activity with intermittent weeks of fewer cases. Both outbreaks also had approximately 10 % of the "asymptomatic" controls confirmed as positive for CHIKV infection in laboratory tests. Interestingly, approximately 8 % of the suspect cases reported having hemorrhagic manifestations. Given the lack of laboratory testing through most of the country, it is easy to understand how CHIKV outbreaks could easily be mistaken for dengue. Although this would be considered atypical for CHIKV infection, previous outbreaks in Thailand (Burke et al. 1985) and Myanmar (Thein et al. 1992) also showed similar levels of hemorrhagic fever. One other commonality between the two outbreaks was that there were dramatic increases in the amount of rainfall leading up to the initiation of the outbreaks. This link to seasonal increased rainfall has also been reported with previous CHIKV activity in Thailand (Thaikruea et al. 1997) whereas in Asian areas where rainfall is less seasonal (Halstead 1966) or in Africa where drought preceded CHIKV epidemics (Chretien et al. 2007), cases have been reported to occur at any time during the year.

For almost the next decade, numerous small foci of CHIKV illness were reported from multiple islands across much of Indonesia. Case counts were never above 5000 in any location but lack of reporting and diagnostics combined with logistical challenges may have led to underestimates of the scope of each of these events (Kosasih et al. 2013). Febrile illness studies performed in Bandung, Java during 2000–2008, but not specifically associated with any outbreak, provided an estimated CHIKV infection incidence rate of 10.1/1000 persons/year with nearly 7 %

of the febrile episodes due to CHIKV (Kosasih et al. 2013). Overall, the number of cases identified during the course of the study (2000–2004 and 2006–2008) remained relatively consistent over the years with cases being identified year round. The study included follow-up serology over 2 years and revealed that IgM antibodies persisted for 3–22 months and IgG titers peaked at 3–4 months post illness but persisted at high titers for up to 2 years following infection. This study also found only the Asian genotype in all samples sequenced even though the ECSA genotype had been identified in other regions of Southeast Asia beginning in 2006. Curiously, in the first phase of the study (2000–2004), arthralgia was not particularly prominent with only 38 % of the patients exhibiting this symptom. In contrast, 87 % of the individuals had arthralgias in the 2006–2008 cohort which is much more typical during investigated CHIKV outbreaks. However, the first cohort was not specifically asked about the presence of arthralgia so the percentage reporting this particular symptom could have been an underestimate. Overall, these nonoutbreak infected individuals exhibited mild infections with one third missing no work and one third missing only 1–3 days. One significant finding of this study was that many CHIKV cases in endemic regions were not associated with large outbreaks but rather were found throughout the year in affected regions without any apparent clustering. Whether severe disease is linked specifically to outbreaks and milder illness is associated with endemic transmission remains an important topic to be further evaluated. Additionally, the importance of this ongoing endemic activity throughout Indonesia would be realized in late 2013 on a small Caribbean island.

Global Expansion

Although the dramatic movement of CHIKV from Kenya throughout the Indian Ocean, India, and Southeast Asia from 2004 to 2010 was previously unprecedented, the virus was still constrained to the Eastern hemisphere. The most significant global expansion of CHIKV distribution occurred from 2011 to 2014 when outbreaks occurred in the western Pacific, the South Pacific, the Caribbean, and the Americas from Florida to central Brazil.

The year 2011 saw the expansion of CHIKV to New Caledonia (Cao-Lormeau and Musso 2014). The number of cases was small, but the arrival of the virus there was not surprising given the movement of the virus around Southeast Asia (Roth et al. 2014a). What was unexpected was the determination that the genotype associated with these cases was the Asian genotype rather than the broadly circulating ECSA Indian Ocean lineage. However, because the first two cases were travelers who had recently been in Indonesia and because the Asian genotype was circulating in Indonesia (Mulyatno et al. 2012), the finding of the Asian genotype was not unreasonable. This Asian genotype continued to be detected across the Western and South Pacific islands with activity in Papua New Guinea in 2012, the Federated States of Micronesia in 2013, Tonga, Samoa, American Samoa, Tokelau, and numerous islands of French Polynesia in 2014 (Roth et al. 2014b), and Kiribati

and the Cook Islands in 2015 (Nhan and Musso 2015; Musso et al. 2015). Interestingly, molecular epidemiology suggests that the virus was actually not just circulating among these islands but was likely reintroduced to the area from affected areas in the Americas and Asia. For example, the Yap outbreak appears to have been initiated by travelers from the Philippines (Lanciotti and Valadere 2014) where a large outbreak was on-going whereas strains characterized from cases in French Polynesia were genetically more similar to isolates from the Caribbean than from nearby Tonga (Aubry et al. 2015). Identifying these movement patterns further depicts the ease of global movement of arboviral pathogens and demonstrates the value of rapid molecular characterization to identify high-risk areas (Powers 2011).

While CHIKV was quietly moving throughout the Pacific Ocean islands, it very noticeably began autochthonous transmission in the Caribbean in late 2013. Transmission in the Western hemisphere was first documented on the island of St. Martin in December (Leparc-Goffart et al. 2014). Before the year ended, three other islands, Guadeloupe, Martinique, and St. Barthelemy, also reported local cases. At the time, awareness of the prevalence of the Asian genotype activity was unappreciated and initial assumptions were that the ECSA genotype, which was still broadly circulating in India and Southeast Asia, had finally made its way to the Americas (Powers 2015b).

The speed with which the virus moved throughout the Caribbean islands was startling with stepwise progression of transmission being reported in virtually every country within just 9 months demonstrating the intensity of movement between the islands. Within the first year of the virus presence in the Americas, 26 island countries and 14 mainland countries were affected with nearly one million cases reported (Powers 2015a). Although activity in many of the island countries has declined or ceased completely, countries in Central and South America continue to report increasing activity suggesting the virus has indeed become endemic in the Americas. At just under 2 years of transmission in the Western hemisphere, PAHO reports approximately 1.6 million cases in 45 countries (PAHO 2015). Interestingly, the vast majority of the cases in the Americas are Asian genotype, but the ECSA genotype (not the Indian Ocean lineage, however) has also been identified in central Brazil (Teixeira et al. 2015). The genetic evidence links this cluster to strains in Gabon and clearly demonstrates that at least two introduction events in the Americas have resulted in establishment of localized transmission.

Conclusions

CHIKV has had an interesting historical journey from its initial discovery, to enzootic pathogen with opportunistic and sporadic infections, to urban epidemic agent, and finally to global vector-borne virus. The range of CHIKV now covers all tropical and some subtropical areas of the globe encompassing the same distribution as pathogens such as dengue viruses. Even with this tremendous number of at-risk individuals, the concern due to CHIKV is still not high, likely due to its lack of mortality.

However, the rapid global expansion of CHIKV provides a lesson for what will come. There are literally hundreds of vector-borne viruses that could move worldwide within a very short period of time, and there will be more that will follow the path that CHIKV has taken. A global surveillance network is critical for monitoring movement of zoonotic pathogens and preparing for the next such introduction event.

Disclaimer The findings and conclusions in this report are those of the author and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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Vaccines Against Chikungunya Virus Infection

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Introduction

Since the first isolation of Chikungunya virus (CHIKV) in Tanzania in the 1950s (Robinson 1955) sporadic outbreaks were found to emerge in various locations in Africa and Asia. However, following an adaptation to the globally distributed tiger mosquito *Aedes albopictus* (Tsetsarkin et al. 2014) CHIKV had larger outbreaks in the Indian Ocean area in 2005 and this was followed by other outbreaks in Asia involving millions of cases (Suhrbier et al. 2012). Furthermore, due to increased traveling worldwide, CHIKV has also spread to nonendemic areas such as Europe (Italy and France), Australia, the Americas, and Polynesia (Johansson 2015; Johansson et al. 2014; Powers 2015). With the occurrence of autochthonous

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outbreaks in nonendemic areas CHIKV infection has now emerged as a global concern (Rougeron et al. 2014; Weaver and Lecuit 2015).

Currently there is no therapeutic treatment of CHIKV infection and although passive immunotherapy has been shown to be efficacious in animal models and could serve as one alternative (Couderc et al. 2009) this approach is yet to be tested in humans. There is currently no licensed vaccine available that could prevent CHIKV infection and given the expanding incidence of CHIKV infections globally, many teams have become engaged in the development of a CHIKV vaccine. This quest has recently been reviewed elsewhere (Ahola et al. 2015; Garcia et al. 2015; Garcia-Sastre and Mena 2013; Powers 2014; Weaver et al. 2012) and thus here we mostly focus on new developments and in particular on results emerging from recent clinical studies.

Novel Vaccine Candidates

Live Attenuated Vaccines

As live attenuated viruses in general have proven to be highly efficacious (Plotkin et al. 2013; Plotkin and Plotkin 2011), a number of CHIKV candidate vaccines have been developed using this approach. Two virus strains with large deletions in either the nsP3 gene or covering the entire 6K gene (Table 1) were shown to generate robust long-term B- and T-cell immune responses after a single immunization and to protect mice fully from a very high dose challenge with a wild-type CHIKV strain (Hallengärd et al. 2014a). Serial passage of the deletion mutant viruses demonstrated that they were genetically fully stable over ten passages and maintained their phenotypes.

In another approach, attenuated mutants were generated via nine amino acid deletions of the *trans*-membrane domain of the envelope protein E2 and by selecting host range (HR) mutants. This approach led to reduction in virus titers, however, it provided a safety feature as these viruses could readily replicate (be produced) in insect cells but did not propagate well in mammalian cells. This vaccine displayed no reactogenicity in mice and generated good humoral responses that were protective against CHIKV challenge (Piper et al. 2013).

While the first two attenuated vaccine candidates were administered as virus particle preparations, different approaches of delivery were employed by placing the complete CHIKV encoding regions of the ΔnsP3 and Δ6K replicative mutants under the control of the cytomegalovirus (CMV) promoter. When these DNAs were delivered by intradermal electroporation robust immune responses were obtained that included both binding and neutralizing antibodies. The potency of these vaccines was similar to those delivered as virus particles. The strategy was to allow productive replication of the attenuated viruses by delivery of naked DNA in order to circumvent the need to grow large quantities of CHIKV in cell cultures (Hallengärd et al. 2014a). A similar approach (denoted iDNA strategy) was taken where the genome of

Table 1 Chikungunya vaccine candidates

Vaccine Type	Platform/Strategy	CHIKV Antigen	References
Live attenuated virus	TSI-GSD-218 (181/clone25)	Full-length	Edelman et al. (2000); Hoke et al. (2012)
	E2 position 79	Full-length	Gardner et al. (2014)
	ΔnsP3	Replicating virus with deletion in nsP3	Hallengärd et al. (2014a)
	Δ6K	Replicating virus lacking 6K gene	Hallengärd et al. (2014a)
	HR mutant/ΔTMD	Full-length with truncation mutation in *trans*-membrane domain	Piper et al. (2013)
	CHIKV/IRES	Replicating virus with IRES sequence	Chu et al. (2013); Plante et al. (2011); Roy et al. (2014a)
Inactivated virus	Formalin inactivated virus	Wild-type virus	Kumar et al. (2012); Tiwari et al. (2009)
Virus-like particles (VLPs)	Production in baculovirus infected insect cells	C-E3-E2-6K-E1	Metz et al. (2013a); Wagner et al. (2014)
	Produced from DNA transfected HEK293	C-E3-E2-6K-E1	Akahata and Nabel (2012); Akahata et al. (2010); Chang et al. (2014a); Noranate et al. (2014)
Subunit vaccines	Bacterially expressed antigen	E1; E2	Khan et al. (2012)
	Bacterially expressed antigen	E2	Kumar et al. (2012)
Chimeric alphaviruses	VEE/EEE/CHIKV	Full-length chimera	Wang et al. (2011b); Wang et al. (2008)
Recombinant viruses	Adeno/CHIKV	Adenovirus expressing C-E3-E2-6K-E1	Wang et al. (2011a)
	VSV/CHIKV	Venezuelan equine encephalitis virus expressing E3-E2-6K-E1	Chattopadhyay et al. (2013)
	MV/CHIKV	Measles virus expressing C-E3-E2-6K-E1	Brandler et al. (2013); Ramsauer et al. (2015)
	MVA/CHIKV	Modified vaccinia Ankara virus expressing C- E3-E2-6K-E1	Garcia-Arriaza et al. (2014)
		Modified vaccinia Ankara virus expressing E3-E2	Weger-Lucarelli et al. (2014)
		Modified vaccinia Ankara virus expressing E3-E2-6K-E1, E3E2, or 6K-E1	van den Doel et al. (2014)
Plasmid DNA	DNA	C-E2-E1; nsP2	Mallilankaraman et al. (2011); Bao et al. (2013)
	DREP Δ5nsP3	Full-length cDNA of CHIKV genome with deletion in nsP3 under pCMV promoter. Produces replicating virus upon transfection	Hallengärd et al. (2014a)
	DREP Δ6K	Full-length cDNA of CHIKV genome lacking 6K gene under pCMV promoter. Produces replicating virus upon transfection	Hallengärd et al. (2014a)
	DREP-ENV	cDNA of CHIKV genome lacking capsid gene under pCMV promoter	Hallengärd et al. (2014b)
	iDNA; 181/clone 25	Full-length cDNA of CHIKV with two attenuating mutations in E2 (T_{12}>I, G_{82}>R) under pCMV promoter. Produces replicating virus upon transfection	Gorchakov et al. (2012); Tretyakova et al. (2014a)

Abbreviations: *HR* host range, *TMD trans*-membrane domain, *VLP* virus-like particle, *IRES* internal ribosome entry site, *HEK* human embryonic kidney, *DREP* DNA replicon

the attenuated strain 181/25 strain (TSI-GSD-218) of CHIKV was expressed from the CMV promoter and delivered as a DNA vaccine (Tretyakova et al. 2014).

In a third approach, Gardner and coworkers subjected the CHIKV strain 181/25 to several passages on evolutionary divergent cell types and generated attenuated variants with increased electrostatic potential in their attachment proteins (Gardner et al. 2014). One particular virus mutant was identified as displaying a new mutation in the E2 protein at position 79 (Table 1). These kinds of approaches may be valuable in the search for attenuated vaccine strains carrying multiple mutations to avoid reversions.

One of the most advanced attenuated vaccine candidates is a strain derived from the La Réunion 2006 outbreak isolate. It was engineered to carry an internal ribosome entry site (IRES) element between the nonstructural and the structural genes to attenuate the virus and to prevent it from replicating in the transmitting *Aedes* mosquito host. The vaccine was tested in several mouse models and induced good levels of neutralizing antibodies and protected them from challenge (Chu et al. 2013; Plante et al. 2011). It also resulted in cross-protective immunity against O'nyong-nyong virus in mice (Partidos et al. 2012). This vaccine and a novel variant were recently tested in nonhuman primates where they demonstrated strong immunogenicity without signs of disease. Both vaccine candidates prevented viremia upon challenge with wild-type CHIKV (Roy et al. 2014). The CHIK-IRES vaccine is now projected for phase I clinical trials by Takeda Inc.

In yet another approach chimeric viruses between CHIKV and the TC-83 strain of Venezuelan equine encephalitis (VEE) or eastern equine encephalitis (EEE) viruses were constructed. These strains were attenuated, replicated well in cell culture, and induced robust protective immune responses in mice (Wang et al. 2008, 2011b). However, it is unclear whether these vaccine candidates remain in the clinical development pipeline.

Recombinant Viral Vectors

A number of strategies have focused on using nonalphavirus vectors for expression of CHIKV antigens. Accordingly, an adenovirus vector was constructed carrying the structural polyprotein gene cassette of CHIKV. The vaccine completely protected mice from viremia and arthritis after challenge with the La Réunion and Asian isolates (Wang et al. 2011a). This vaccine candidate was in the development pipeline of GenPhar Inc., however, as the company is no longer in business it is unclear whether this vaccine will ever be evaluated clinically. Other recombinant viruses constructed include a vesicular stomatitis virus VSVΔG-CHIK with the glycoprotein (G) gene replaced by the entire CHIKV structural polyprotein gene cassette (Chattopadhyay et al. 2013) and a recombinant measles virus MV-CHIK also expressing the structural proteins of CHIKV (Brandler et al. 2013). Both vaccines generated robust immune responses and protected mice from lethal challenge. The MV-CHIKV vaccine was constructed using the MV platform developed

by Institute Pasteur and has recently been evaluated in a clinical trial (see below under "Clinical Trials"; Ramsauer et al. 2015).

A recombinant poxvirus-CHIK vaccine candidate based on the modified vaccinia virus Ankara (MVA) strain expressing all of the CHIKV structural genes (C-E3-E2-6K-E1), triggered robust B- and T-cell immune responses with high protection efficacy (Garcia-Arriaza et al. 2014). In addition, two slightly different MVA-CHIKV vaccine candidates have also been developed. The first one expresses the E3-E2 (p62 precursor) proteins and although protective immune responses were obtained in a mouse challenge model, the vaccine candidate was not very immunogenic (Weger-Lucarelli et al. 2014). This is probably because the E3-E2 proteins were not expressed on the cell surface and thus would not be efficient in stimulating antibody responses to E2. As p62 needs to complex with E1 in the endoplasmic reticulum to allow efficient transport of the heterodimeric E2-E1 envelope spike complex to the cell surface this would explain the lack of surface exposure. This was corroborated by the fact that p62 was not cleaved to E3 and E2 which is the action of the furin protease in the *trans*-Golgi compartment during spike transport to the cell surface. Furthermore, because p62 was not processed and as it was not in complex with E1 one would expect the E2 moiety to have an incorrect conformation as compared to wild-type virus. Collectively, the observed protection against challenge must be contributed to linear epitopes in the E3E2 proteins or perhaps in part be T-cell mediated.

In yet another study recombinant MVA vaccines expressing E3-E2, 6K-E1, or E3-E2-6K-E1 were evaluated (van den Doel et al. 2014). The vaccines induced protection against CHIKV challenge, however, the MVA expressing 6K-E1 only protected up to 75 % of the animals. Overall it seems that the MVAs that did not express the full-length CHIKV polyprotein cassette were not very immunogenic. The reason for this would be the same as discussed above for the E3-E2 MVA, however, it is difficult to understand why the E3-E2-6K-E1 vaccine was not more immunogenic, at least in comparison with C-E3-E2-6K-E1 (Garcia-Arriaza et al. 2014). This could be related to the removal of three immunomodulatory viral genes acting on the interferon system that have been shown to enhance immune responses to MVA expressed antigens (Garcia-Arriaza and Esteban 2014). Neither vaccine induced formation of VLPs (the E3-E2-6K-E1 vaccine would not be expected to) but both expressed functional spike envelope complexes on the cell surface. Although the MVA-E3-E2-6K-E1 vaccine induced neutralizing antibody titers around 40–160 the MVA-C-E3-E2-6K-E1 vaccine did so with titers in the range of 4000–10,000. The capsid protein could carry a dominant epitope (Hoarau et al. 2013; Kam et al. 2012a), however, this would only help in protection against challenge but would not be, in the absence of VLP formation, a factor concerning levels of neutralizing antibodies. Clearly there are differences in the methods to measure neutralizing antibody titers, one being a NT assay based on reduction/suppression of CPE (van den Doel et al. 2014) and the other a virus replicon particle- based NT assay (Gläsker et al. 2013), however, they should not differ more than threefold (Gläsker et al. 2013). This raises the question of what exact role binding antibodies and T cells may play in protection against CHIKV infection.

DNA Vaccines

In addition to attenuated CHIKV or vaccines vectored by other viruses the use of DNA vaccines to combat CHIKV has also been pursued. One of the first was a DNA vaccine expressing the envelope proteins E3, E2, and E1 (Muthumani et al. 2008). Later an improved version of this vaccine employed a C-E2-E1 construct that was found to generate neutralizing antibodies protecting mice against virus challenge. The vaccine was also tested in nonhuman primates where neutralizing antibody responses were obtained after five immunizations employing electroporation as a means for enhancing delivery. However, the vaccinated animals were never challenged with wild-type CHIKV (Mallilankaraman et al. 2011). The same investigators also investigated the use of the CHIKV nsP2 gene as an adjuvant in an attempt to improve the protective capacity of a CHIK DNA-Env vaccine. It was claimed that immune responses were improved and led to better protection in virus challenge experiments (Bao et al. 2013).

As a variation to the DNA vaccine theme, a replicon-based DNA (DREP) vaccine expressing the CHIKV replicase and envelope proteins E3-E2-6K-E1 was developed. This vaccine carries the complete genomic region of CHIKV, however, lacking the gene coding for the capsid protein. Thus upon delivery this replicon will replicate its RNA in the same fashion as a wild-type or attenuated CHIKV, but it will not be able to produce new virus particles as nucleocapsids cannot form, hence there is no viral spread in tissue. This is an important safety feature of this vaccine. The DREP vaccine was shown to be highly immunogenic generating robust B- and T-cell immune responses (Hallengärd et al. 2014b).

Protein (Virus) Vaccines

Attempts have been pursued with the goal of developing subunit vaccines or vaccines based on inactivated whole virus preparations. One such study utilized bacterially produced rE2 and rE1 protein antigens delivered in combination with a number of different adjuvants. Balanced Th1/Th2 immune responses were obtained including generation of neutralizing antibodies. However, the mice were not challenged in this study (Khan et al. 2012). In a separate report also using bacterially produced rE2 antigen a number of adjuvants were tested. Although good immune responses were obtained with protection from challenge not all adjuvants were effective (Kumar et al. 2012).

Two studies have been involved in testing formalin-inactivated whole virus preparations that were grown on monkey Vero cells. In both studies Th1/Th2 balanced humoral responses were obtained. Although one study did not perform challenge studies (Tiwari et al. 2009) the other could demonstrate good protection in a mouse model (Kumar et al. 2012). It is unclear whether these vaccine approaches are in the clinical pipeline. In a similar approach, a vaccine against Ross River virus (RRV) was recently tested in a randomized phase III trial and was found to be well tolerated and immunogenic (Holzer et al. 2011; Kistner et al. 2007; Wressnigg et al. 2015).

However, based on an expected effective neutralizing titer of >1:10, three doses of this formalin-inactivated vaccine were needed to achieve >80 % seroconversion (91.5 % responders in the 16–59 age group and 76 % in the >60 age group). That two immunizations only result in 30 % seroconversion suggests the immunogenicity of this vaccine needs improvement.

Virus-Like Particles (VLPs)

The use of virus-like particles (VLPs) is an interesting development given the success with other VLP-based vaccines. CHIKV-derived VLPs have been shown to be morphologically, antigenically, and immunologically similar to native CHIKV (Akahata et al. 2010; Noranate et al. 2014; Wagner et al. 2014). One approach for producing VLPs involved the transfection of human embryonic kidney cells (HEK), 293 cells with plasmid DNA encoding the CHIKV structural proteins C-E3-E2-6K-E1 under the CMV promoter. Stability studies on these VLPs have been performed resulting in recommendations for long-term storage of parenteral formulations (Kramer et al. 2013). These VLPs proved to be immunogenic in nonhuman primates where they induced good levels of neutralizing antibodies that were protective against a stringent challenge with wild-type virus (Akahata and Nabel 2012; Akahata et al. 2010). Protection was mainly antibody dependent as passive transfer of serum into naïve mice rendered these animals immune against challenge. This VLP vaccine has completed a phase I clinical trial (see below under "Clinical Trials") (Chang et al. 2014a). It will be important and interesting to assess how this technology will perform in terms of production yields and stability (Kramer et al. 2013).

Another similar approach engaged the infection of insect cells in culture with recombinant baculovirus expressing the structural gene cassette of CHIKV (Metz et al. 2013a). Immunization of mice with nonadjuvanted VLPs resulted in the generation of high levels of neutralizing antibodies and provided complete protection against challenge. The same group further demonstrated that VLPs were more immunogenic than corresponding subunit antigens E1 or E2 that were produced in insect cells (Metz et al. 2011, 2013b). These findings are in line with results coming from studies using recombinant MVA for expressing E1 and E2.

Due to limitations in scale-up production of VLPs in HEK293 cells or in baculovirus-infected insect cell cultures, an improved production method was recently developed. This employs high-pH adapted *Spodoptera frugiperda* insect cells that resulted in a tenfold increase in production yields. The resulting VLPs seemed to be equally immunogenic in guinea pigs when compared to the HEK293-produced VLPs (Wagner et al. 2014). Insect cell production appears to be the method of choice, but yields and cost are still open questions. The improved production yields using baculovirus vectors now approach those using HEK293,that is, about 10 μg per 10^8 infected cells. With an estimated yield after downstream processing of 10 % (Wagner et al. 2014) and an assumed human dose of 20 μg given in a 3-dose regimen (see clinical trial results below) it would require 6×10^{15} infected cells to produce one million human doses.

Prime-Boost Studies

Optimally, the goal in the development of an efficacious CHIKV vaccine is to have a vaccine that could raise long-term protective immunity after a single immunization. This is particularly so as a CHIKV vaccine would not be expected to be part of any national immunization program. Moreover, although humoral immunity seems to play a central role in protection, we do not fully understand the correlates of protection in the case of CHIKV disease. Therefore, in the absence of a vaccine it is of value to compare different vaccine candidates in single vaccine modalities or in heterologous prime-boost regimens. Prime-boost combinations are known to enhance immune responses and can be used to focus the immune response in an advantageous way. A recent study evaluated several vaccine candidates in prime-boost regimens (Hallengärd et al. 2014b). Comparing immune response to those obtained during wild-type CHIKV infection, live attenuated CHIKV (Hallengärd et al. 2014a), DNA replicon (DREP), and MVA-CHIKV (Garcia-Arriaza et al. 2014) were used as priming vaccines followed by booster immunizations with either the homologous vaccine or by a heterologous MVA-CHIKV or E2E1 protein antigen vaccine. The protein antigen (Voss et al. 2010) was a covalently linked dimer of the E2 and E1 envelope proteins of CHIKV and was comixed with the adjuvant Matrix-M (Magnusson et al. 2013).

Immune responses (binding antibodies) against a live attenuated virus vaccine (ΔnsP3, see Table 1) could be enhanced by giving the same virus vaccine as a boost (5× enhancement) or by boosting with a protein (E2E1) antigen (10×). However, when the virus vaccine and the protein antigen were given at the same time (but in different body locations) immune response was only enhanced 5×. Interestingly, the Th1/Th2 balance (IgG2c/IgG1) remained always in favor of Th1 (as consistently was found for ΔnsP3 alone), thus the addition of the protein with a Th2-promoting adjuvant had little effect on the balance. Similarly, when a DNA replicon vaccine (DREP, Table 1) was used as a prime, the immune response could not be enhanced over homologous prime-boost regimens unless the protein antigen was given sequentially. Again the priming with the replicon maintained the Th1 over Th2 balance. When a DREP vaccine was boosted with an MVA-CHIKV vaccine significant enhancement was observed but addition of a protein antigen to the same regimen had now an advantageous effect. Finally, using the MVA-CHIKV as a prime followed by a boost with the protein antigen did not result in stronger immune responses whereas administration of two doses of MVA-CHIKV was better than giving it only once. As was the case with the other two priming agents, Th1/Th2 balance was maintained, indicating that priming with a virus vector/vaccine will prime the immune system to maintain its Th1/Th2 balance.

T-cell responses in general followed the same pattern as seen for the humoral responses. Virus and replicons generated robust antigen-specific CD8 T-cell responses that, however, were not significantly enhanced by addition of the protein antigen. Importantly, the combination of using DREP prime followed by MVA-CHIKV boost resulted in a tenfold enhancement of T-cell responses (1000 vs. 10,000 spot forming units, SFU).

Immunobiology of CHIKV Vaccines

To date, all the vaccine candidates developed have, with the exception of three, only been tested in animals. Inasmuch as almost all the candidates induced good protective immune responses it is difficult to draw strong conclusions as to the merits of each candidate. Moreover, it is difficult if not impossible to determine correlates of protection that could drive design and testing of future vaccine candidates in the clinical pipeline. For example, the exact roles of antibodies and T cells in protection against CHIKV infection and/or chronic disease have yet to be clearly defined. Studies have certainly suggested that antibodies play an important protective role against acute infection (Fric et al. 2013; Goh et al. 2013; Pal et al. 2013; Selvarajah et al. 2013). Thus, although adoptive transfer of T cells did not confer protection (Chu et al. 2013), passive transfer of immune sera did so (Chu et al. 2013; Plante et al. 2011; Akahata et al. 2010). Furthermore, vaccines inducing mainly CD8-specific T cells did not protect whereas vaccines inducing neutralizing antibodies did (Mallilankaraman et al. 2011). However, a recent study showed that T cells do play a role in suppression of viremia, although this occurs secondary to antibodies and IFN α/β (Poo et al. 2014). In this instance CD4 T cells (rather than CD8 T cells) are implicated in this antiviral activity (Poo et al. 2014; Teo et al. 2013). In addition, CD4 T cells are also associated with arthritic disease in CHIKV infection (Nakaya et al. 2012; Teo et al. 2013). Most important, these preclinical results are strongly supported by findings from natural CHIKV infections in humans.

Protective humoral immune responses in CHIKV-infected human patients are mainly targeted against the E2, E3, and nsp3 protein antigens. Interestingly, although the production of early neutralizing IgG3 antibody levels was associated with high levels of viremia during the early disease phase, patients with such clinical manifestations recovered fully. On the other hand, patients with low levels of viremia showing slower development of IgG3 responses appeared to stand a higher risk of developing chronic arthralgia (Kam et al. 2012a, c).

A number of linear and nonlinear epitopes have been associated with neutralization (or escape therefrom) with most of them being located in areas of the receptor binding domain in E2, in E2 areas that interact with the E1 fusion loop or result in disturbance of the functions of the E1 fusion loop. Identification of such domains and epitopes will give guidance for monitoring and design of vaccine candidates. A potent human neutralizing monoclonal antibody C9 isolated from an individual who recovered from CHIKV infection defines an E2 epitope that includes residue A162 within the acid-sensitive region (ASR). The ASR has been shown to be involved in spike rearrangements during fusion and viral entry and is key in the structure-function of the spike complex. Although the C9 antibody binds to the outer and top edge of the trimeric spike complex another human monoclonal antibody designated E8 (involving residues Y69, F84, V113, G114, T116, D117) binds to the central part of the spike and appears not to be neutralizing (Selvarajah et al. 2013). Furthermore, the epitope V216 in the E2 domain B (distal ectodomain that may interact with the cellular receptor) and amino acid residue T101 in E1 (fusion groove) have been defined by the two broadly neutralizing antibodies 5F10 and 8B10, respectively. It is

important to note that these sites were also shown to be involved in escape from neutralization resulting in cell-to-cell transmission of CHIKV (Lee et al. 2011; Fric et al. 2013). The E2EP3 epitope (STKDNFNVYK) at the N-terminus of E2 and proximal to the E3E2 furin cleavage site was shown to be a dominant linear epitope that strongly associated with virus neutralization (Kam et al. 2012b). In corresponding murine studies the residue K252 was demonstrated to be involved in the stabilization of the envelope complex (Akahata and Nabel 2012). Separately, monoclonal antibody CHK-152 was shown to protect the fusion loop of E1 and CHK-9, m10, and m242 antibodies define the receptor-binding site (Sun et al. 2013). Recently, in an extensive study involving phage display, several monoclonal antibodies were isolated against CHIKV envelope proteins E2 and E1 (Fong et al. 2014). Epitope mapping using shotgun mutagenesis revealed, among others, a conformational epitope (IM-CKV063) strongly involved in neutralization. This epitope spans two E2 subunits in the envelope spike complex such that residues E24 and I121 are in one subunit whereas residues G55, W64, K66, and R80 reside on an adjacent subunit. All epitopes that reside on the topmost and outer surface of the E2/E1 trimer are neutralizing whereas those facing the interior of the trimer are not. A recent study of nonhuman primates revealed that unique linear epitopes were recognized in the CHIKV-infected animals. However, two major epitopes were identical in NHPs and in humans (the E2EP3 epitope in position 2800–2818 and another epitope in position 3025–3056). However, the strong neutralizing noncontiguous epitope IM-CKV063 was identified in the human material but not in the NHPs (Kam et al. 2014).

Clinical Trials

Three clinical studies of CHIKV vaccines have been evaluated to date. The three studies differ quite extensively with regard to their approach.

Attenuated CHIKV Virus Vaccine

An early and conceptually important strategy was the development of an attenuated vaccine candidate. This was based on a clinical isolate originating from Thailand in 1962. The US Army Medical Research Institute of Infectious Disease (USAMRIID) passaged this strain in human MRC-5 cells, which resulted in an attenuated strain named TSI-GSD-218 or 181/clone25 (Levitt et al. 1986; Hoke et al. 2012; Table 1). This vaccine candidate showed promising results in phase I (McClain et al. 1998) and phase II (Edelman et al. 2000) clinical trials. The vaccine was highly immunogenic and a single dose of this attenuated virus was sufficient to achieve 98.3 % seroconversion (69 % by day 14 and 98.3 % by day 28). One year later 85 % of the volunteers still had neutralizing antibodies against CHIKV. Geometric mean titers on day 28 were 1:852 (max 1:10.240) and had declined to 1:105 (max 1:1.280) by day 360. Despite these good results the further development of this vaccine was discontinued partly

because of side effects (arthralgia in 8 % of volunteers) and also because of uncertainties about the production process (Hoke et al. 2012). Low market interest can also have contributed to the withdrawal. Of note, a recent study showed that 181/25 is only attenuated by two point mutations in the E2 envelope glycoprotein suggesting that reversions may occur (Gorchakov et al. 2012). This finding puts safety into question for this vaccine. Indeed, it may be that the reason for the observed arthralgia in 8 % of the volunteers perhaps was not the reactogenicity of the vaccine preparation but rather presence of a significant amount of revertant (wild-type) particles.

Virus-Like Particle Vaccine (VLPs)

The vaccine VLP vaccine candidates developed by the NIH group (Akahata and Nabel 2012; Akahata et al. 2010) was recently tested in a clinical phase I trial (Chang et al. 2014b). The trial was a dose-escalation open-label trial with altogether 23 volunteers receiving three doses of 10, 20, or 40 μg of VLPs. The results showed the VLP vaccine to be well tolerated with no serious adverse events reported. Weak binding antibody responses were detected after the first vaccination (100 % of seroconverted in the 20 μg group and in 80 % in the 10 and 40 μg groups). These antibody responses were boosted after the second immunization but a third immunization was required to achieve peak titers 4 weeks after the third dose. However, this response was significantly reduced by week 24. Induction of neutralizing antibodies followed the same pattern as for binding antibodies, with low responses after the first vaccination and the anamnestic responses after the second and third immunizations. Again neutralizing antibody titers had declined significantly by 24 weeks after the third immunization. It was reported that convalescent neutralizing antibody titers in patients 3 months after disease ranged from 4000 to 7000, which was the same as the level of titers in this clinical trial 4 weeks after the third vaccination. Therefore, although the VLP vaccine is fairly immunogenic in humans, it requires three immunizations to achieve protective antibody levels that are maintained for up to 6 months. Perhaps the future use of adjuvant in combination with the VLPs could enhance immune responses, should this be required

Recombinant MV-CHIKV

The third clinically evaluated vaccine strategy is the use of recombinant measles virus vector expressing the structural proteins of CHIKV (Brandler et al. 2013; Table 1). In a recent phase I dose escalation clinical trial volunteers were immunized with three doses (low, medium, and high) of MV-CHIKV (Ramsauer et al. 2015). After priming the volunteers were either boosted on day 28 or on day 90. After the prime immunization, 40 % of the volunteers in the low dose group seroconverted whereas 92 % and 90 % seroconverted in the medium and high dose groups, respectively. After the second immunization all volunteers had seroconverted. As this is a recombinant measles

virus (MV) vaccine it was important to assess whether pre-existing antibodies against MV would hamper the immune responses against CHIKV antigens. The material is still limited but it appears that pre-existing MV immunity did not play a major role. With regard to safety the candidate MV-CHIKV vaccine had an acceptable tolerability profile. However, 8 % of the volunteers in the medium dose group and up to 25 % in the high dose group displayed severe adverse events. Thus, the medium group had the best ratio of immunogenicity to tolerability. Considering the persistence of antibody responses, all groups required a booster immunization. In the low and medium dose groups antibody titers declined quite rapidly whereas they were somewhat better in the high dose group, the group with the highest percentage of severe adverse events.

Considerations from the Clinical Trials

Ideally, any vaccine should be safe and induce long-term B- and T-cell immune responses with good recall capacity. Therefore, it is of interest to examine the immune responses obtained after immunization with the three CHIKV vaccine candidates evaluated in clinical trials so far. The best measure of protection at this time is levels of neutralizing antibodies. Unfortunately, because of the differences between the assay methods, the neutralization titers obtained after immunization with the different vaccines cannot be directly compared. However, some trends of the immune responses can be seen when the kinetics of the contraction is followed over time (Table 2). The neutralizing antibody response obtained after two immunizations with the MV-CHIKV vaccine seems to contract to about 20 % after 2 months.

Table 2 Neutralizing antibody titers induced in the CHIKV clinical trials

	GMT NT_{50} Titer[a]		
Vaccine	Peak Value[b]	$\text{Memory}_{\text{Day}}$[c]	% Memory[d]
1× TSI-GSD-218[e]	582	240_{120}; 105_{360}	41; 18
2× L MV-CHIKV[f]	73	16_{64}	22
2× M MV-CHIKV[f]	150	28_{64}	19
2× H MV-CHIKV[f]	433	66_{64}	15
3× 10ug VLP[g]	8745	940_{140}	11
3× 20ug VLP[g]	4525	717_{140}	16
3× 40ug VLP[g]	5390	1385_{140}	26

[a]*GMT* geometric mean titer. For the TSI and MV-CHIKV trials variants of the plaque reduction neutralization test (PRNT) were used. For the VLP trial a flow-cytometry–based fluorescence reduction assay was used

[b]Highest antibody titer measured after the first (TSI), second (MV-CHIKV), or third (VLP) immunization

[c]Memory titers measured at indicated day following day of peak titer

[d]Percent neutralizing antibody titer remaining at memory

[e]Attenuated replicating vaccine virus administered once

[f]Recombinant measles virus expressing CHIKV antigens. Vaccine was given twice with low (L) medium (M), or high (H) doses in a prime-boost regimen

[g]Virus-like particle vaccine given three times at indicated low, medium, and high doses

Not surprisingly, the higher the dose administered, the higher is the elicited antibody response. However, the magnitude does not seem to affect the contraction rate of the antibody response (if anything the contraction rate appears to be faster when using the higher dose although this difference is probably not statistically significant). In comparison, three immunizations with the VLP vaccine contracts to about 20 % during the course of 3–4 months. In great contrast to the first two, a single immunization with the attenuated TSI-GSD-218 virus results in antibody responses that decrease to about 40 % of the peak response at 4 months and reach 20 % after 1 year. Clearly, the attenuated vaccine is more efficacious as a single dose with significantly longer memory compared to the other vaccines that need to be given two or three times for effect. Thus, although these are early days, more work certainly will be needed before strong conclusions can be made.

In Summary

It will be important for CHIKV vaccine candidates to address not only the strength and longevity of the specific immune responses but also the quality of the response. In this instance one can expect greater differences between vaccine platform technologies. Further investigation for the establishment of well-defined correlates of protection and the key roles played by innate, adaptive, and memory immune cells triggered by the different vectors will be needed. In addition, safety, stability of the vaccines/vectors, and yields required for manufacture all need to be assessed. The goal will be to have a long-term highly protective vaccine against chikungunya virus infection.

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Function of Chikungunya Virus Structural Proteins

Stefan W. Metz and Gorben P. Pijlman

Introduction

Chikungunya virus (CHIKV) belongs to the genus *Alphavirus* (within the family *Togaviridae*), which includes over 29 species that may cause encephalitis, febrile illness, and arthralgia in humans. CHIKV has a single-stranded, positive sense RNA genome of approximately 12 kb in length, although some size variation exists between different lineages. The icosahedral virions have a diameter of 60–70 nm and consist of a nucleocapsid enveloped by a host-derived phospholipid membrane. The viral structural polyprotein is translated from a ~5 kb subgenomic mRNA and is co- and post-translationally cleaved into capsid protein (C), two major envelope glycoproteins (E1, E2), and three smaller accessory proteins (E3, 6K, and the transframe protein TF). Together, the structural proteins encapsidate the viral genomic RNA to form the viral progeny. The different functions of the individual CHIKV structural proteins in virion assembly, egress, binding, and fusion are reviewed in this chapter.

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Chikungunya Virus Capsid Protein

The CHIKV nucleocapsid has a $T=4$ icosahedral symmetry and consists of the viral RNA encapsidated by 240 capsid protein copies (Fig. 1a, b). The CHIKV capsid protein has a length of 261 amino acids and an apparent molecular weight of ~30 kDa (Khan et al. 2002), which is small enough for passive transport through nuclear pores. The CHIKV capsid protein is organized into 3 regions (I, II, and III) with separate functions (Fig. 1c) (Hong et al. 2006).

The capsid protein is autocatalytically cleaved off *in cis* from the nascent viral structural polyprotein (C-E3-E2-6K-E1) by its C-terminal serine protease which has a chymotrypsin-like fold (Aliperti and Schlesinger 1978; Choi et al. 1991; Melancon and Garoff 1987; Strauss and Strauss 1994). A catalytic serine S213 and several conserved H139, D145, D161 amino acids are predicted to be involved in this autoprotease activity which resides in region III of the capsid coding sequence (Hahn and Strauss 1990; Khan et al. 2002). After the autocatalytic cleavage of the capsid from the structural polyprotein, the signal sequence for ER translocation of PE2 becomes available at the N-terminus.

The capsid has a poorly conserved N-terminal region with an alleged role in viral RNA assembly via positively charged Arg, Lys, and Pro residues in region I. Region II is involved in the encapsidation of newly synthesized viral genomic RNA, which is packaged with high specificity due to defined RNA packaging signals within the nsP2 gene. Consequently, only full-length genomic RNA (and not viral subgenomic

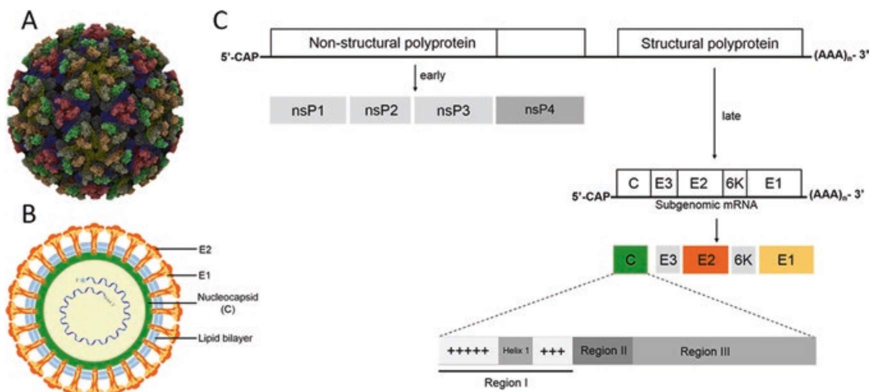

Fig. 1 CHIKV structure and genome. (**a**) CHIKV virion and (**b**) schematic representation of CHIKV particle section (adapted from Metz and Pijlman (2011)). (**c**) Schematic representation of the CHIKV genome, which encodes 2 ORFs. The nonstructural polyprotein encodes nonstructural proteins nsP1, nsP2, nsP3, and nsp4. The structural polyprotein is translated from a subgenomic mRNA and encodes the capsid protein (**c**), envelope glycoproteins E1 and E2 and accessory proteins E3 and **6K**. Capsid is segmented into three separate regions. Region I contains many positively charged residues as well as uncharged amino acids in helix 1. Region II is responsible for RNA binding and region III interacts with the E2 glycoprotein

RNA or host mRNA) is encapsidated in the virions (Owen and Kuhn 1996; Weiss et al. 1989). The capsid protein requires interactions with nucleic acids (i.e., RNA) to initiate assembly.

Alphavirus capsids, including CHIKV, contain an 18 amino acid long coiled-coil α-helix within region I that is important for assembly. Initially, viral RNA-bound capsid dimers are formed, 120 of which further oligomerize to form the nucleocapsid in the cytoplasm of infected cells (Perera et al. 2001). The nucleocapsids are then transported to the plasma membrane for association with the C-terminal cytoplasmic tail of E2 to initiate virion budding. The capsid protein contains a hydrophobic pocket adjacent to the protease substrate binding site within region III for binding to the cytoplasmic endodomain of the E2 glycoprotein, which extends down into the site of the hydrophobic pocket (Kuhn 2007b).

The intracellular localization of the CHIKV capsid is mainly cytoplasmic, although a predicted nuclear localization signal (NLS) is present in the N-terminus (Thomas et al. 2013). Although a putative biological role for a nuclear fraction of CHIKV capsid in viral infection is currently not known, New World alphavirus capsid proteins actively translocate to the nucleus to induce host transcriptional shut-off (Garmashova et al. 2007).

Chikungunya Virus Envelope (Glyco)Proteins

Alphaviruses contain a membraneous envelope that is derived from the infected host cell during budding. Specialized viral glycoproteins embedded in the envelope regulate cell receptor recognition, attachment, and cell entry through fusion of viral with host membranes. CHIKV encodes a single envelope polyprotein comprising four envelope proteins in the order E3-E2-6K-E1. There is also an additional transframe protein (TF) originating from a frameshift event at the 3′ end of 6K (Firth et al. 2008). Not all structural proteins from the envelope cassette are incorporated in progeny virus particles; that is, CHIKV E3 is not generally found associated with virions (Simizu et al. 1984).

The two most conspicuous proteins of the envelope cassette are the glycoproteins E1 (viral fusion protein) and E2 (receptor binding protein). CHIKV-E2 is initially expressed as precursor E2 (E3 E2, or PE2) and matures throughout multiple cellular compartments. Both E1 and E2 are N-linked glycosylated, type I integral membrane proteins that contain transmembrane domains proximate to the C-terminus, followed by a cytoplasmic tail. E1 and E2 heterodimers are exposed at the virion surface as trimeric spikes and consequently are the main targets for a neutralizing antibody response, although E2 is the primary antigen (Strauss and Strauss 1994). In addition to binding cell receptors during the vesicular entry pathway, E2 is essential in intracellular translocation and folding of the other envelope proteins and acts as a stabilizing factor for E1–E2 interactions during transport from the ER to the plasma membrane.

CHIKV E1–E2 Trimer Formation and Virus (-Like Particle) Assembly

The envelope of CHIKV is a multiprotein structure composed of glycoprotein trimeric spikes embedded in a host-derived membrane. The 80 trimeric spikes in the CHIKV virion each consist of three E1–E2 heterodimers and are essential for cell receptor recognition (E2) and entry through pH-dependent endocytosis (E1) (Kielian et al. 2010).

Once capsid is released from its nascent polypeptide chain, the remaining envelope polyprotein or envelope cassette is translocated and inserted into the endoplasmic reticulum (ER) membrane for initial processing (Fig. 2). A series of apolar residues at the N-terminal end of PE2, within the E3 coding region, functions as a signal peptide for the translocation of the envelope cassette into the lumen of the ER (Lobigs et al. 1990). The E3 signal is not processed or removed by host signalases, indicating a distinct role in trimer formation. This was exemplified by the replacement of E3 with an artificial signal peptide that targeted the polyprotein to the ER, but abolished trimeric spike formation and surface expression (Lobigs et al. 1990). The role of E3 in correct translocation and processing of PE2 was also demonstrated by the expression of individual CHIKV-E3E2 protein constructs using both insect and mammalian expression systems (Metz et al. 2011; van den Doel et al. 2014; Voss et al. 2010).

In the ER, proteolytic processing by host signalases cleaves 6K at its N- and C-terminal end, releasing 6K from the envelope polyproteins and yielding PE2, 6K, and E1 (Fig. 2). Immediately after processing, PE2 and E1 form heterodimers, which is followed by glycosylation and the oligomerization of three pE2-E1 dimers to form the immature and nonfusogenic trimeric spike complex (Kuhn 2007a).

Alphavirus E1 and E2 are N-linked glycosylated, but the number of glycans may vary between species (Blom et al. 2004; Burke and Keegstra 1979; Knight et al. 2009; Rice and Strauss 1981; Simizu et al. 1984). CHIKV-E1 is predicted to be glycosylated once at N141 and E2 is glycosylated at N263 and N273 (Blom et al. 2004).

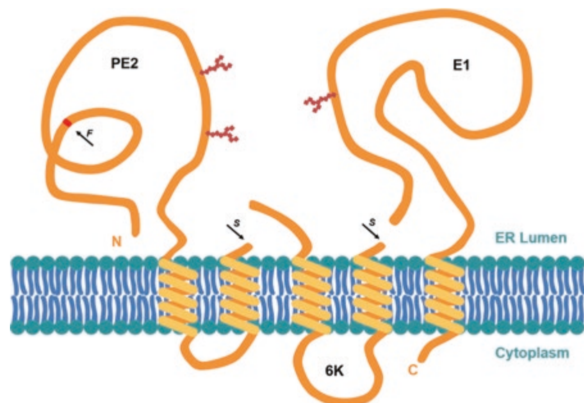

Fig. 2 CHIKV envelope glycoprotein organization. After capsid is released, the envelope cassette is inserted into the ER and subsequently processed by host signalases (S) and furin-like proteases (F). *Blue chains* indicate N-glycosylation sites. Adapted from (Kuhn 2007a)

For the alphavirus type species Sindbis virus it has been shown that glycosylation does not necessary influence processing of PE2 or E1 but does influence virulence and viral replication (Knight et al. 2009).

Alphaviruses use low-pH triggered fusion to facilitate entry of target cells. This process is mediated by fusogenic active trimeric complexes at the surface of the virus particle. It would be detrimental for viral replication if the fusion protein is activated during the formation of the trimeric spike as it encounters the low-pH environment of the secretory pathway. The CHIKV fusion protein E1 is therefore expressed with the companion protein E2. The dimeric interaction between E1 and E2 protects the fusion protein from low-pH-induced premature activation (Uchime et al. 2013). The late secretory pathway involves compartments with a pH ranging from ~5.5 to 6.0, which is sufficient to trigger premature E1 inactivation and fusion (Uchime et al. 2013). However, the immature PE2-E1 dimer is more acid stable than the fully mature E2-E1 form.

E3 plays a critical role in the stabilization and protection of the immature trimer from acidic environments. After initial processing and dimerization, the small peripheral E3 protein is finally released from PE2 by furin-dependent cleavage in the *trans*-Golgi system (Strauss and Strauss 1994). Following cleavage, E3 remains noncovalently associated with the fully processed trimeric spike and is released upon spike surface exposure at neutral pH. Furin processing is not a precondition for CHIKV virion assembly, but incomplete processing results in impaired fusion activity of the immature trimeric spikes (Strauss and Strauss 1994). Crystal structures of both PE2-E1 and E2-E1 heterodimers show that E3 exclusively interacts with E2, suggesting a dimer-stabilizing mechanism to protect E1 from premature activation before it reaches the cell surface (Li et al. 2010; Voss et al. 2010). Mutational studies have shown that the pH-protective interaction between E3 and E2 is highly dependent on a single amino acid (Y47) within the E3–E2 interface (Uchime et al. 2013).

CHIKV has been shown to be very suitable for the generation of so-called virus-like particles (VLPs). These artificial viral particles are replication defective due to absence of genomic viral RNA, but share the morphological properties of wild-type virus (Fig. 3). This means that VLP formation most likely shares all characteristics and processing kinetics found during wild-type CHIKV replication.

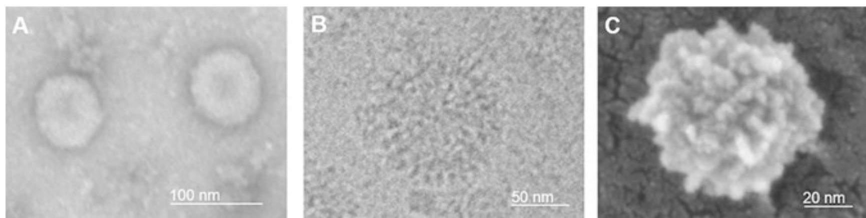

Fig. 3 CHIKV virus-like particles produced in insect cells. (**a**) CHIKV VLPs visualized by transmission electron microscopy after negative stain, (**b**) cryo-electron microscopy, and (**c**) scanning electron microscopy

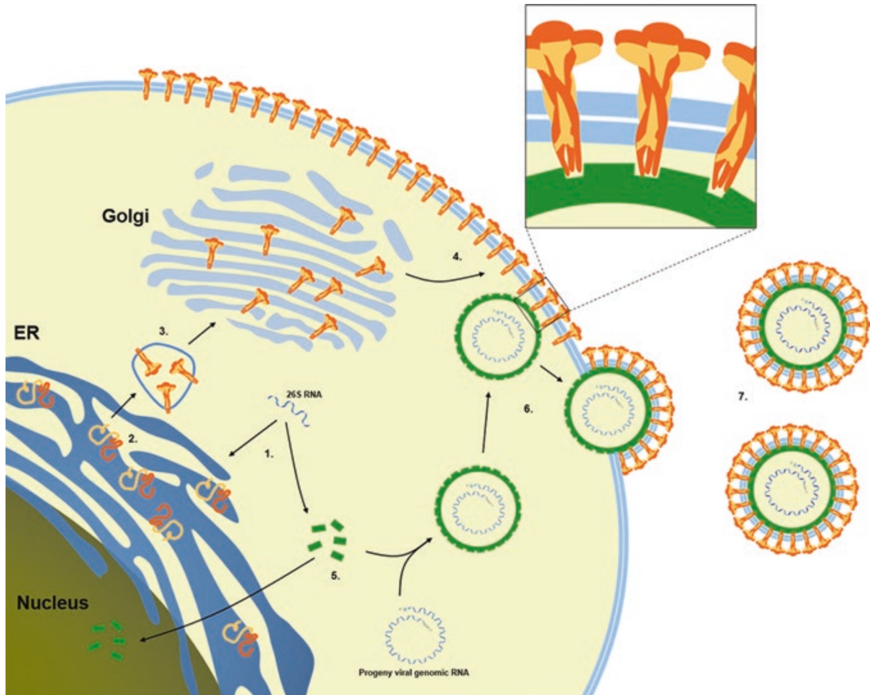

Fig. 4 CHIKV virus particle formation and maturation. The CHIKV structural proteins are translated from the 26S RNA (*1*) after which the envelope glycoproteins E1 and E2 are transported to the ER, complex into heterodimers (*2*) and are transported to the Golgi (*3*) where three heterodimers complex into trimeric spikes and furin processing takes place. (*4*) The mature trimeric spikes are transported to the plasma membrane and are exposed on the surface of the cell. The capsid protein interacts with the progeny viral genomic RNA and assembles into nucleocapsids in the cytoplasm (*5*). The nucleocapsids bud out from the plasma membrane, taking along the trimeric spikes anchored in the lipid bilayer (*6*), resulting in mature CHIKV particles (*7*)

For the production of CHIKV VLPs, the complete structural polyprotein is expressed within a mammalian or insect cell expression system to allow correct poly(glyco)protein processing and trimer formation (Fig. 4) (Akahata et al. 2010; Metz et al. 2013a; Noranate et al. 2014).

Interestingly, different CHIKV strains yield variable quantities of VLPs. It has been shown that particle assembly and release are related to factors such as palmitoylation of the E1 and E2, cholesterol requirements, and pH-levels. A so-called acid-sensitive region (ASR) has been identified in E2, which is known to initiate conformational changes in the E1–E2 complex. Mutational changes of especially amino acid 234 in E2, or changes in pH increased VLP production yields.

In many neutralizing antibody escape mutants, modifications of the E2 B-domain have been found, in addition to modifications in the E2-ASR. Specific amino acid substitutions have been identified that prevent antibodies from neutralizing the virus by binding to amino acids that regulate the conformational changes to prime to

fusion complex active (Coffey and Vignuzzi 2011). All these characteristics can be used to optimize VLP production yields, especially because CHIKV-VLPs are considered to be among the most promising vaccine candidates in development (Akahata et al. 2010; Metz et al. 2013a, b).

Accessory Proteins 6K and Transframe Protein TF

For decades it was believed that there were only five structural proteins encoded by CHIKV (C, E3, E2, 6K, and E1) and other alphavirus species. 6K is a small, hydrophobic acylated protein that is involved in membrane permeabilization and envelope development but rarely is incorporated into mature virions (Antoine et al. 2007; Firth et al. 2008; Strauss and Strauss 1994; Welch and Sefton 1979). The C-terminal domain of 6K acts as an ER-translocation signal for E1. However, 6K deletion mutants have shown that E1 still localizes to the ER by the N-terminal signal sequence of PE2 (Liljestrom and Garoff 1991). Thus, envelope glycoprotein translocation can act independently of 6K (Metz et al. 2011; Strauss and Strauss 1994).

In the ER, 6K is processed by host signalases and released from PE2 and E1 after which 6K becomes associated with the PE2–E1 complex and is transported with the complex to the cell surface. Yet, during virus budding, 6K is mostly excluded from integration into new virons (Lusa et al. 1991). Even though 6K deletion mutants are still viable, mutations in 6K are associated with decreased virion production with impaired fusion activity and core deformations (Antoine et al. 2007; Firth et al. 2008; Gaedigk-Nitschko et al. 1990; Gaedigk-Nitschko and Schlesinger 1990; Loewy et al. 1995). Several studies have shown the importance of 6K in virus budding. It is postulated that 6K allows lipids from the membrane to flip from one side of the bilayer to the other (Gaedigk-Nitschko et al. 1990). With the use of chimeric alphaviruses it was found that 6K interacts in a sequence-specific manner with PE2 or E1. This interaction is required for efficient virus budding because 6K proteins are not interchangeable between alphavirus species (Yao et al. 1996).

Early observations report that the 6K migrated as a doublet, and differences found in protein size and biochemical properties were explained by different levels of acetylation. Recent analyses of this 6K doublet and its coding region resulted in the identification of a sixth structural protein. This so-called transframe protein (TF) originates from a ribosomal −1 frameshifting event at the C-terminus of the 6K coding region (Firth et al. 2008). This frameshifting occurs at an estimated efficiency of approximately 10–18 % and takes place at a conserved UUUUUUA motif within the 6K coding sequence. The resulting TF of approximately 8 kDa in size shares its N-terminus with 6K, but lacks the second transmembrane region found in 6K. The TF C-terminus is encoded by the −1 frame. Additional alphavirus 3′sequences involved in efficient frameshifting have a remarkable diversity (Chung et al. 2010).

6K has been shown to possess viroporin properties, a small protein that is able to increase membrane permeability that favors virion budding. Interestingly and other than 6K, TF has transmembrane domain flanking regions that are rich in basic resi-

dues, which is characteristic for several other viroporins. Thus, TF may be the actual viroporin and therefore important for virus budding (Firth et al. 2008). Further analysis revealed, though in rare occasions, that not 6K but TF is predominantly incorporated in viral particles, suggesting a role in the formation and budding of new virions (Firth et al. 2008).

Similar to 6K, TF is not absolutely required for genome replication or envelope protein translocation to the cell surface, but abolishing its production severely decreases virus particle release in both mammalian and insect cell systems (Snyder et al. 2013). Even though the precise mechanisms underlying their roles in membrane permeabilization, ion-gradient formation, and virus assembly and release are not yet fully understood, 6K and TF are both critical players in the late stages of CHIKV virion formation.

Role of CHIKV Structural Proteins During Viral Entry and Fusion

In general, alphaviruses are able to infect a wide range of species and cell types, because the viruses are most likely able to recognize and bind a range of different receptors on the host cell surface (Kielian et al. 2010). The process of particle attachment and absorption is a multistep event orchestrated by CHIKV-E1 and E2. Being an arbovirus, CHIKV infects both insect and vertebrate cells, meaning that the virus needs to deal with a wide range of divergent biochemical and genetic environments. Thus it is likely that CHIKV uses ubiquitous receptors and/or is able to bind multiple (protein) receptors (Kononchik et al. 2011).

Virus infection starts with scanning cell surfaces to encounter one or more suitable receptors. Subsequent binding of the virus is saturable and is primarily mediated by E2 (Kielian et al. 2010). Even though the exact receptors have not been identified yet, several proteinaceous or polysaccharide molecules have been suggested to be attachment factors for alphaviruses. The list includes the high affinity laminin receptor (Wang et al. 1992), heparin and heparan sulfate (Byrnes and Griffin 1998; Smit et al. 2002; Zhang et al. 2005), heat shock 70 protein (Ryan et al. 1992), the major histocompatibility complex (Helenius et al. 1978; Maassen and Terhorst 1981), and DC-SIGN and L-SIGN (Klimstra et al. 2003). The latter ones are C-type lectins that have high binding affinity for mannose-rich carbohydrate structures. Interestingly, mosquito cells produce these high mannose structures and mosquito-derived virus shows increased binding and infection on DC-SIGN/L-SIGN expressing cells. Thus, a high mannose glycan state of E2 might have a strong influence on its ability to bind receptor-associated molecules (Klimstra et al. 2003).

Upon receptor binding by E2, conformational changes within the trimeric spike and the mild acidic endosomal environment trigger the dissociation of the E1–E2 heterodimer and induce a homotrimerization event between the three E1 molecules. CHIKV-E1 is a type II fusion protein and its ectodomain is composed of three domains: the central domain (DI), the fusion loop containing domain (DII), and

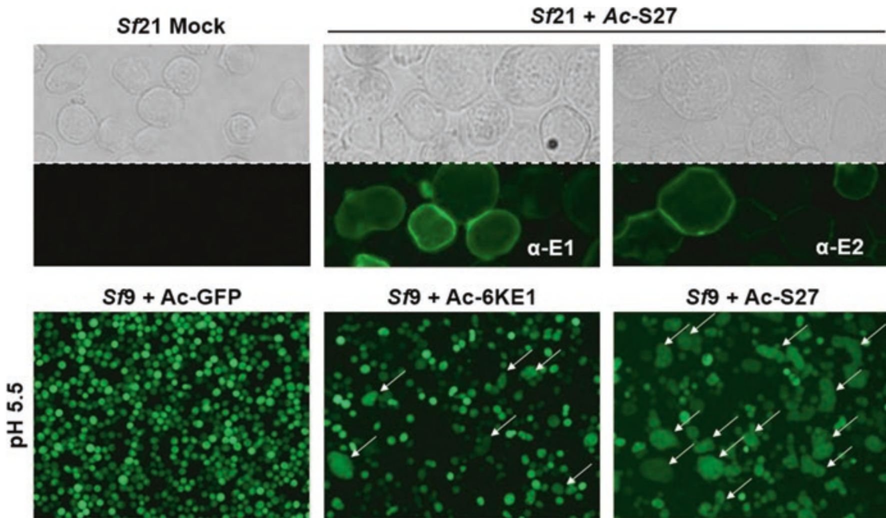

Fig. 5 CHIKV glycoprotein expression and fusogenicity. The complete structural protein cassette of CHIKV was expressed in insect cells using recombinant baculoviruses. (**a**) Healthy and infected Sf-21 insect cells were immunostained with E1 and E2 specific antibodies. The clear ring-like structures indicate surface expression of the glycoproteins. (**b**) Individual E1 and the complete structural cassette of CHIKV were expressed in GFP-expressing insect cells. At a pH of 5.5, the fusion protein E1 is activated, resulting in the fusion of proximate cell membranes known as syncytia formation

domain III (DIII), which connects to the transmembrane domain (TM; Sanchez-San Martin et al. 2013). The E1 fusion loop is located at the tip of DII and is normally shielded in the E1–E2 interface by interaction with histidine residues in a so-called fusion loop binding groove within E2 (Voss et al. 2010). During fusion, E1 is inserted into the host's plasma membrane through the fusion loop. A core trimer is formed by interactions between DI and DII, forming a hairpin-like structure by which DIII is able to pack against the core trimer. This conformational reassortment brings the viral and target membrane in close proximity enabling membrane fusion (Gibbons et al. 2004). Even though the subtle interactions between E1 and E2 are critical in the formation of the surface trimers, E1 on itself remains fusogenic (Metz et al. 2011). When E1 is expressed in a 6KE1 context in insect cells, syncytia are formed between E1-expressing cells that display E1 on the cells' surface (Fig. 5).

In the past decade, more evidence has accumulated that supposes an alternative entry mechanism for alphaviruses independent of endocytosis, exposure to low pH, and membrane fusion. E1-mediated membrane fusion is a so-called nonleaky process where viruses are taken up into the cell without losing plasma membrane continuity. The alternative entry mechanism is supported by the fact that infection by alphaviruses appears to be a leaky process that allows the passage of ions and small molecules across the compromised membrane (Kononchik et al. 2011; Koschinski et al. 2005; Madan et al. 2005; Wengler et al. 2003, 2004). Such ion-permeable pores are believed to be formed by E1 insertions into the viral or E1-expressing cell

membrane, in the absence of a target membrane (Koschinski et al. 2005). The importance of these ion-permeable pores in infection is still being discussed, yet again it shows the critical role of E1 in virus entry.

In addition, research is now focusing on the potential role of 6K or TF in the pore entry pathway. Even though the two small proteins are rarely incorporated in the virus particle, they are able to generate plasma membrane pores late in infection and 6K/TF deletion mutants generate viable, but fusogenically compromised virus particles (Sanz et al. 2003).

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Functions of Chikungunya Virus Nonstructural Proteins

Tero Ahola and Andres Merits

Introduction

The nonstructural proteins (nsPs) of chikungunya virus (CHIKV) and other alphaviruses are encoded at the 5′ region of the positive-strand viral RNA genome, and thus translated directly after the entry of the RNA into the cytoplasm, to enable the immediate start of the RNA replication process. Many of the replicative functions remain poorly studied for CHIKV nsPs, but have been earlier characterized for proteins from other alphaviruses, primarily Semliki Forest virus (SFV) and Sindbis virus (SINV; Kaariainen and Ahola 2002). It is expected that the basic biochemical functions are conserved in CHIKV proteins, the amino acid (aa) sequence of which are on average 71 % identical with the more closely related SFV nsPs. Therefore we review data from CHIKV whenever available, but also draw heavily on other alphaviruses, and point out the uncertainties regarding the functions of CHIKV proteins.

Secondly, the nsPs have many functions in host–virus interactions, including the evasion of antiviral responses. Clearly, these functions are more virus- (and/or cell-type) specific in nature, so care needs to be taken in making inferences regarding CHIKV. For instance, it has been shown that hnRNP K, a cellular protein identified as a pro-viral (essential) factor for CHIKV replication (Bourai et al. 2012) acts as a negative effector of replication for SFV (Varjak et al. 2013). A primary division within the mammalian/avian alphaviruses lies between the Old World viruses (e.g., CHIKV, SFV, and SINV) and the New World viruses (e.g., Venezuelan equine encephalitis virus, VEEV), and these two groups display quite different modes of

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host interaction (Garmashova et al. 2007; Kim et al. 2016). Due to the multiple functions of the nsPs, a complex pattern of protein localization is observed within the cell. A sizable fraction of the nsPs stays together in the replication complexes, but a significant fraction also displays localizations specific to each of the proteins, engaging in various virus–host interactions.

For CHIKV itself, most of the data we have concerning the functions of the ns-proteins originates from studies of proteins encoded by viruses belonging to the East/Central/South African (ECSA) genotype (typically from the Indian Ocean outbreak of 2005–2007). However, two more genotypes of CHIKV, West African (WA) and Asian, are known. Ns-polyproteins, encoded by viruses belonging to different genotypes, have 95 % (WA compared to the other two genotypes) to 97 % (ECSA compared to Asian genotype) identity. The identity is highest in nsP4 and lowest for the C-terminal hypervariable region of nsP3. Three to five percent difference in aa sequence certainly creates a possibility that some of the nonenzymatic functions of nsPs encoded by different CHIKV genotypes may be substantially different. Thus far, no head-to-head comparison of functions of nsPs from different CHIKV genotypes has been performed. However, comparison of data available for WA and ECSA genotype viruses supports the possibility that the nsPs encoded by viruses from these genotypes have substantially different properties in specific host interactions (see below under “nsP2”).

Expression and Processing of Nonstructural Polyproteins

In alphaviruses, the replication proteins are simply called nonstructural proteins 1-4 (nsP1-4). Similar to many other positive-stand RNA viruses, the replicase proteins of alphaviruses are expressed in the form of ns-polyprotein precursor(s) (Strauss and Strauss 1994). Most clinical isolates of CHIKV encode for two ns-polyproteins, P123 and P1234; the latter results from the read-through of an in-frame opal termination codon located close to the sequence encoding for the C-terminus of nsP3. However, in some CHIKV isolates and laboratory-adapted strains, the opal codon is replaced by a codon for arginine and the corresponding genomes express P1234 as the only ns-polyprotein. Interestingly, the presence or absence of the opal codon seems to have relatively minor effects on the virus, most likely because in natural isolates of the virus both variants (with the terminator and without it) of the genomes are present. As in the other alphaviruses, the expression of CHIKV ns-polyprotein(s) occurs only at the early stages of infection in mammalian cells and is shut down typically 6–8 h post infection (Scholte et al. 2013), due to the general inhibition of cellular translation during infection.

In order to give rise to functional proteins, the processing of ns-polyproteins must be precise and well regulated. CHIKV P1234 contains three cleavage sites hereafter termed 1/2 (site between nsP1 and nsP2), 2/3, and 3/4 sites (Fig. 1). All three sites are processed by the viral protease activity residing in nsP2 or in ns-polyproteins containing nsP2. The protease must specifically recognize each of the cleavage sites, and different affinity towards the three sites is partially responsible

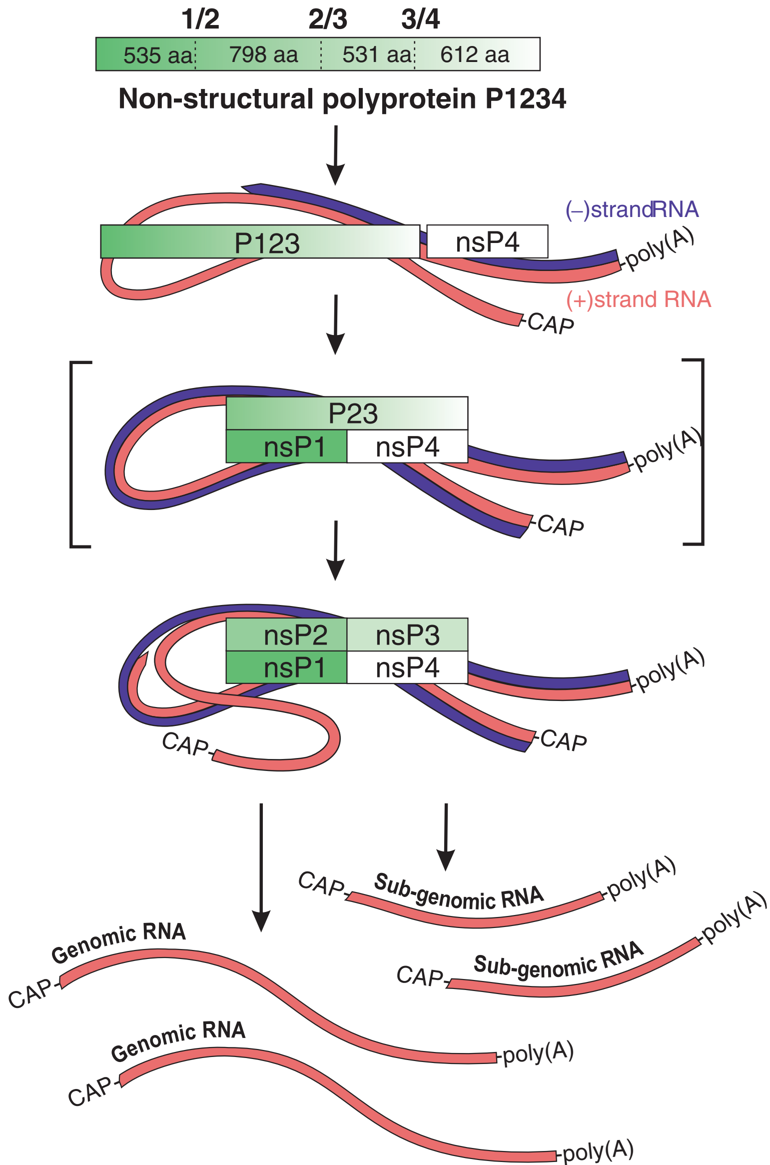

Fig. 1 Processing of CHIKV ns polyprotein P1234 and RNA synthesis. P1234 is shown at the top with the cleavage sites and sizes of the individual nsPs indicated. The intermediate P123 + nsP4 is responsible for negative-strand synthesis, the nsP1 + P23 + nsP4 complex is very short-lived (shown in brackets), and mature replication complexes producing positive-strand genomic and subgenomic RNAs consist of fully processed nsPs

for the ordered pattern of cleavages (Lulla et al. 2006). However, recent studies of SFV P1234 processing reveal that a second mechanism, the presentation of cleavage sequences via long-range interactions between different domains of the polyprotein,

has a major role (Lulla et al. 2012, 2013). The three cleavage sites of CHIKV ns-polyproteins are more similar compared to each other than those of SFV, therefore one can assume that for CHIKV the presentation of cleavage sites has the same or even a larger role than for SFV. Current data already indicate that the cleavage patterns of P1234 in CHIKV and SFV are closely similar: in order to allow the formation of functional replicase complexes (see below), the cleavage of the 3/4 site must take place first, followed by cleavage of the 1/2 and finally by cleavage of the 2/3 site (Vasiljeva et al. 2003).

Replication Cycle

After the cleavage of the 3/4 site and recruitment of the viral genome as template, the partially processed replicase P123 + nsP4 is responsible for negative-strand synthesis (Lemm et al. 1994; Shirako and Strauss 1994; Wang et al. 1994; Fig. 1). Replication complexes cannot be assembled from fully processed nsPs, but instead the polyprotein P123 is an essential intermediate. It is hypothesized that normally each replicase would make only a single negative strand and then switch to positive-strand synthesis, with the newly made negative strand as the template. This switch is connected to the processing of 1/2 and 2/3 sites, and replication complexes active in positive-strand synthesis thus contain a set of fully processed nsPs (Fig. 1). If these two latter cleavages are blocked, positive-strand synthesis still occurs but is very inefficient (Lemm et al. 1994; Shirako and Strauss 1994). The final cleavage of the 2/3 site may further facilitate the synthesis of subgenomic RNAs. CHIKV expresses a single subgenomic RNA, whose synthesis is initiated from the subgenomic promoter located on the negative strand. The subgenomic RNA corresponds to the final third of the genomic RNA, and it encodes the structural proteins of the virus. Both positive-strand RNAs are 5′ capped and 3′ polyadenylated. This model of RNA synthesis, although attractive and verified with other alphaviruses, has some unsolved conundrums. First, the stoichiometry of functional replication complexes remains unknown, and they may contain multiple copies of some or all of the nsPs or their precursors, as suggested, for example, by the *trans*-cleavage of the 2/3 site (see below under "nsP2"). Secondly, replication is connected with the formation of small membrane invaginations known as spherules as the site of replication (see the concluding section), and therefore the geometry of the replication complexes could be strictly constrained in a limiting space.

Recently, a robust in vitro replication system derived from CHIKV-infected cells has been described, which should be amenable to biochemical analysis, and it is also very useful in characterizing the mode of action of antiviral molecules (Albulescu et al. 2014). A *trans*-replication system for CHIKV, in which a template and the replication proteins are expressed from separate plasmids, following the model of SFV, has also been constructed (Spuul et al. 2011; Utt et al. 2016).

As a consequence of polyprotein processing, only nsP1 has an N-terminal Met residue whereas the N-terminal residues of nsP2, nsP3, and nsP4 of CHIKV are Gly,

Ala, and Tyr, respectively. The native N-termini represent important requirements for the functionality of nsP2, nsP4, and possibly nsP3 in different alphaviruses: the properties of these proteins are usually altered, and some of their functions may even be abolished by minor changes such as the addition or substitution of a single N-terminal aa-residue (Vasiljeva et al. 2003; Shirako and Strauss 1998). The same is clearly the case for CHIKV nsP2 (Das et al. 2014b) and presumably also for nsP3 and nsP4. Most likely this property originates from the fact that nsPs in their mature form have partially different functions and/or are involved in different interactions compared to the corresponding regions in ns-polyproteins. This has important implications for the analysis of the functions of alphavirus nsPs; in order to express the proteins, an initiation codon is often added to the expression constructs of nsP2, nsP3, and nsP4 or the proteins are tagged at their termini. Based on the considerations highlighted above, the data obtained by using native (not tagged or truncated) nsPs should most closely reflect the true functions of the proteins, whereas the use of modified proteins can support misleading conclusions. This does not mean that data obtained using such tools have no value; however, researchers and readers should be aware of the biological effects of such manipulations.

nsP1

The enzymatic functions of alphavirus nsP1 are needed in the capping of the viral positive-strand genomic and subgenomic RNAs, which bear a cap0 structure $m^7G(5')ppp(5')ApNp$…. SFV nsP1 was characterized as a unique mRNA capping enzyme, which first transfers a methyl group from *S*-adenosyl-methionine (AdoMet) to position 7 of GTP, and subsequently forms a covalent complex with m^7GMP, releasing pyrophosphate (Ahola and Kaariainen 1995). Finally, the methylated guanylate residue needs to be transferred to the 5′ end of the viral RNAs bearing diphosphate (ppRNA), to complete the cap structure. The first reaction of capping, the triphosphatase step removing the first phosphate of the nascent pppRNA, is carried out by nsP2, as explained below. The reactions catalyzed by nsP1 are unique, inasmuch as cellular and DNA poxvirus-encoded mRNA capping enzymes first transfer GMP to the RNA, and the methylation of the cap is carried out as a final step by a separate cap methyltransferase protein/domain (Decroly et al. 2012). The capping domain related to nsP1 is found in all members of the alphavirus-like superfamily, which encompasses the animal alpha-, rubi-, and hepeviruses as well as dozens of genera of plant viruses, but related proteins have not been found elsewhere (Rozanov et al. 1992). The capping functions of nsP1 are essential for alphavirus replication (Wang et al. 1996). Although the capping activities have been described for SFV and SINV enzymes, given the conservation of function within the entire superfamily, there is no doubt that they are also present in CHIKV nsP1. Due to its unique features, nsP1 is an appealing target for specific antivirals. However, the lack of structural information and the propensity for membrane association (see below) have impeded studies of the protein, and only recently have specific inhibitors been described (Delang et al. 2016).

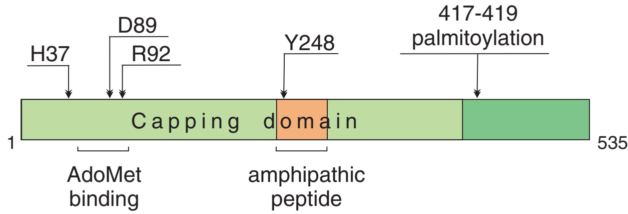

Fig. 2 Schematic of the domain structure of CHIKV nsP1. Conserved active site residues and regions important for membrane association have been marked. See the text for details

Recent sequence and secondary structure analyses have demonstrated that the conserved capping domain is a region of ≥400 aa residues (Ahola and Karlin 2015; Fig. 2). There are only four amino acid residues, which are (almost) universally conserved in all members of the superfamily. A conserved histidine (H37 in CHIKV nsP1) is thought to be the covalent binding site for m^7GMP, a conserved aspartate (D89) is involved in binding the methyl donor AdoMet, and a conserved arginine (R92) and tyrosine (Y248) are also essential for the methyltransferase function of nsP1. Several other residues that are partially conserved and important for function have also been identified (Ahola and Karlin 2015). It has been speculated, based on secondary structure and very limited sequence conservation, that at least the AdoMet-binding site of nsP1 could be structurally related to the large family of methyltransferases having a so-called Rossman fold (Ahola et al. 1997). This hypothesis can only be verified by the high-resolution structure of the protein, which is still lacking. The evolutionary origins of the protein remain an interesting mystery, and could even be a case of virus-specific evolution, where an ancestral methyltransferase has also acquired the ability to catalyze the guanylyltransferase reaction.

nsP1 is the sole membrane anchor of the alphavirus replication complex. The protein does not possess transmembrane segments, but instead interacts with membranes in a monotopic fashion, that is, with only the cytoplasmic sheet of the membrane bilayer. The main interaction appears to be mediated by a membrane-binding peptide located in the central part of the protein (approximately residues 244–263 in CHIKV nsP1; Fig. 2), which forms an amphipathic alpha helix, as characterized for the corresponding peptide from SFV (Ahola et al. 1999; Lampio et al. 2000). The hydrophobic facet of the helix interacts with the hydrophobic lipid chains, whereas the hydrophilic, predominantly positively charged facet interacts with the head groups of the phospholipids. The peptide and the entire nsP1 have a specific affinity towards membranes bearing a negative charge, carrying, for instance, a high density of phosphatidylserine (Ahola et al. 1999). Several residues of the peptide are essential for the membrane binding of the entire replication complex, and perturbation of membrane association is lethal for the virus (Spuul et al. 2007). Sequence comparisons within related proteins from other superfamily members have sug-

gested that there might be other membrane association sites, but these remain to be tested experimentally (Ahola and Karlin 2015).

nsP1 is also covalently modified by palmitoylation, which serves as a secondary mechanism further tightening the membrane association (Laakkonen et al. 1996). nsP1s from different alphaviruses have 1–3 cysteine residues around amino acid 420, CHIKV having three (aa residues 417–419). Although palmitoylation is important for the normal function of nsP1, it has been possible to select SFV variants that are devoid of palmitoylation, but bear compensatory mutations elsewhere in nsP1 (Zusinaite et al. 2007).

When expressed alone in mammalian cells, nsP1 is completely membrane associated and localizes to the cytoplasmic surface of the plasma membrane. In infected cells, nsP1 also directs the entire replication complex to the membrane (Spuul et al. 2007). In some alphavirus infections (e.g., SINV), the replication complexes remain predominantly at the plasma membrane, whereas in others (e.g., SFV) they are later efficiently internalized and found on the outer surfaces of enlarged endosomes and lysosomes (so-called cytopathic vacuoles type I; Frolova et al. 2010; Spuul et al. 2010). nsP1 does not appear to mediate the internalization process, and processed nsP1 molecules not associated with replication complexes remain on the inner surface of the plasma membrane. CHIKV seems to follow the model in which the replication complexes mostly remain on the plasma membrane in mammalian cells (Thaa et al. 2015).

In the replication complex, nsP1 must naturally interact with the other nsPs, but these interactions remain poorly understood. Nevertheless, there is some evidence that a major interaction is with nsP4 (Zusinaite et al. 2007; Fata et al. 2002; Shirako et al. 2000). Interactions of nsP1 with host proteins are also poorly characterized, but similar to the other nsPs, they are important for viral pathogenesis. Interestingly, CHIKV nsP1 downregulates the expression of and thus counteracts the function of tetherin (also known as BST-2), which is a virus restriction factor retaining virus particles on the cell surface (Jones et al. 2013).

nsP2

nsP2 is the largest of the ns-proteins (798 aa residues in CHIKV) and is often said to consist of an N-terminal RNA helicase and C-terminal protease domains. Strictly speaking, this is not correct. The three-dimensional structure of the fragment corresponding to the C-terminal part of CHIKV nsP2 (aa residues 471–791) has been resolved at 2.4-Å resolution (Protein Data Bank code 3TRK), revealing a fold highly similar to that of nsP2 protease from VEEV (Russo et al. 2006) and SINV (Shin et al. 2012). This clearly shows that this fragment consists of two structural domains, a papain-like protease domain (aa residues ~471–605), and a remnant of an Ftsj methyltransferase-like (MTL) domain (aa residues ~ 606–791) that is nonfunctional as a methyltransferase because of the absence of a number of crucial

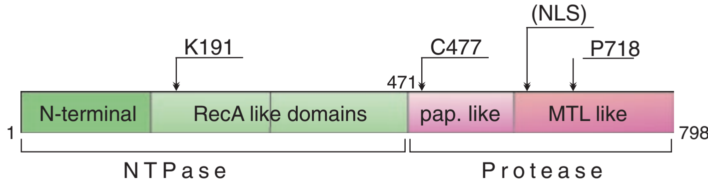

Fig. 3 The domain structure of nsP2, showing the helicase motif I with a conserved lysine and the active site cysteine of the protease. The putative NLS and the conserved proline residue affecting cytotoxicity have been marked

structural elements. These domains function as one unit and they are essential for the protease activity of nsP2. The structure of the N-terminal region of nsP2 has not been resolved. Molecular modeling reveals that it consists of three structural domains, the second and third being RecA-like domains characteristic for RNA helicases. The first N-terminal domain has little homology outside of alphaviruses (Das et al. 2014b). Thus, it is more correct to say that CHIKV nsP2 is a multidomain protein (Fig. 3).

Similar to the other ns-proteins, nsP2 is a multitasking protein having several enzymatic activities and a large array of important nonenzymatic activities. As nsP2 from Old World alphaviruses is central in the suppression of cellular transcriptional and antiviral responses (Garmashova et al. 2006, 2007; Breakwell et al. 2007), CHIKV nsP2 has been studied in more detail than the other CHIKV ns-proteins, and it may already be the best-studied alphaviral nsP2.

Enzymatic functions. nsP2 has four known enzymatic activities, all of which have been revealed and analyzed for CHIKV nsP2.

The first function to be analyzed was the protease activity (nsP2 belongs to papain-like Cys-proteases). It was shown that a truncated recombinant protein, corresponding to aa residues 422–639 of nsP2, has no protease activity. In contrast, a protein spanning from aa residue 422 to the end of nsP2 was capable of cleaving short (3–8 aa residues) fluorogenic peptides corresponding to 1/2 and 3/4 sites (Pastorino et al. 2008). The peptides corresponding to the 3/4 site were cleaved more efficiently than peptides corresponding to the 1/2 site; however, this could be attributed to the differential solubility of substrates. Thus, the true cleavage requirements and efficiencies remained unknown. The actual protease activity of CHIKV nsP2 has been recently analyzed using the setup previously developed for the SFV protease (Lulla et al. 2006, 2012; Vasiljeva et al. 2001): using full-length nsP2 with the native N-terminus as an enzyme and recombinant proteins containing cleavage sites of different length, as substrates (Utt et al. 2015). This analysis revealed that substrates representing 1/2 and 3/4 sites (10 aa residues preceding and 5 aa residues following the cleavage position) were cleaved very efficiently but the substrate representing the 2/3 site was not cleaved at all. This site was, however, cleavable in an extended form (10 preceding and 170 following aa residues), similar to what had been previously shown for SFV. Thus, the cleavages of the 2/3 and 3/4 sites of

CHIKV are very similar to those of SFV. In contrast, whereas SFV protease poorly cleaves the 1/2 substrate in *trans*, CHIKV protease does it very efficiently. In cells at early stages of CHIKV infection both 3/4 and 1/2 sites can be (and likely are) cleaved in *cis*, whereas the 2/3 site is always cleaved in *trans*. Nevertheless, the data indicate that in order to maintain the correct sequence and timing of ns-polyprotein processing, CHIKV needs to rely more on high-order regulation mechanisms and less on sequence-specific recognition of cleavage sites (see above).

The NTPase and RNA 5′-triphosphatase activities of CHIKV nsP2 were reported few years later (Karpe et al. 2011). The assays were carried out using a fusion protein consisting of maltose-binding protein followed by a fragment of nsP2 (aa residues 166–630). Such recombinant protein efficiently hydrolyzed GTP, dGTP, ATP, dATP, and to some extent also CTP, dCTP, and UTP substrates; in contrast no hydrolysis of dTTP was detected. The enzyme also removed γ-phosphates from RNA substrates. As this reaction was significantly inhibited by the presence of ATP it was concluded that the NTPase and RNA 5′-triphosphatase activities have a common active site (Karpe et al. 2011). Interestingly, it was found that unlike many studied helicases belonging to superfamily 1 (SF1), the NTPase activity of the fusion protein was not stimulated by RNA or DNA oligonucleotides. This and other open questions were subsequently addressed in experiments using different forms of recombinant nsP2, including full-length nsP2 with the native N-terminus, full-length nsP2 with a hexahistidine tag at its N-terminus and a truncated N-terminal fragment of nsP2 (aa residues 1–470) (Das et al. 2014b). These recombinant proteins were capable of hydrolyzing all the canonical dNTPs (including dTTP) and NTPs with no clear preference for substrate. The NTPase activity was found to depend on the intactness of the N-terminus of the enzyme as well as on the presence of the C-terminal region: compared to modified enzymes, the native nsP2 was five- to sevenfold more active. Finally, it was demonstrated that the NTPase activity of nsP2 is clearly stimulated by both RNA- and DNA oligonucleotides. This stimulation was, however, dependent on the presence of the C-terminal region of nsP2, explaining why this effect was not detected using a fusion protein lacking aa residues 631–798.

Based on the sequence analysis of the N-terminal 470 aa residues, CHIKV nsP2 belongs to the SF1 group of helicases. Nonetheless, no helicase activity was detected for the recombinant protein used by Karpe and coworkers (Karpe et al. 2011) or for the N-terminal fragment of nsP2 (Das et al. 2014b). In the latter study it was found that only full-length nsP2 is capable of acting as a helicase. nsP2 was unable to unwind double-stranded (ds)DNA or RNA duplexes lacking a 5′ single-stranded overhang. However, it demonstrated unwinding of dsRNA (containing 12 b or longer 5′ single-stranded overhang) in a 5′–3′ directionally biased manner. Furthermore, it was found that nsP2 also has RNA strand annealing activity. Both RNA-helicase and RNA-strand annealing activities were dependent on the presence of the C-terminal region of nsP2. The combined results indicate that functional cross-talk between different domains of nsP2 is essential for the enzymatic activities of the protein (Das et al. 2014b).

Nuclear transport, cytotoxic effects, and interference with antiviral responses. nsP2 is the only ns-protein, which at least in the Old World alphaviruses is localized both to the cytoplasm and the nucleus of infected cells. This phenomenon has been known for a long time (Peranen et al. 1990); however, the mechanism(s) of how nsP2 enters the nucleus is not fully understood. Neither of the two nuclear localization signals (NLS) predicted for SINV nsP2 affects the nuclear transport of the protein (Frolov et al. 2009). In SFV nsP2, a putative NLS has been identified at the beginning of the MTL domain. Mutation of this sequence indeed abolishes the nuclear transport of nsP2 (Rikkonen et al. 1992), however, this is the case only at 37°C but not at 28°C (Tamm et al. 2008). Therefore it is possible that the region is not acting as a canonical NLS, but instead mutations at this site may alter the conformation of the protein in a temperature-dependent manner. In addition, no NLS can be predicted in the corresponding region of CHIKV nsP2 (hereafter the segment of CHIKV nsP2 corresponding to the NLS of SFV nsP2 is referred to as "putative NLS"). Nevertheless, the nuclear localization of nsP2 is not only well documented but also biologically highly relevant, as it is required (but not sufficient) for the inhibition of cellular antiviral responses (Breakwell et al. 2007) and for the shutdown of host–cell transcription. In SINV and CHIKV it has been shown that the latter occurs due to the ability of nsP2 to induce degradation of Rpb1, a catalytic subunit of cellular RNA polymerase II. Unlike the proteases of picornaviruses, which inhibit cellular translation and transcription by cleavage of host cell proteins, the protease activity of nsP2 is not essential for the suppression of cellular transcription (Bourai et al. 2012; Akhrymuk et al. 2012). The ability to induce cellular transcription shutdown is reduced by mutations in the C-terminal region of CHIKV nsP2 (including Pro718 to Gly mutation; Bourai et al. 2012) and by the corresponding mutation in SINV nsP2 (Akhrymuk et al. 2012). However, the effects of mutations introduced into the NTPase/helicase active site were different for these viruses: in SINV such a mutation strongly reduced the nsP2-dependent degradation of Rpb1 whereas CHIKV nsP2 mostly retained its ability to block host gene expression.

The nuclear localization of CHIKV nsP2 is best studied for CHIKV belonging to the ECSA genotype. Immunofluorescence microscopy reveals that in infected cells nsP2 does not localize uniformly. Instead, a lot of variation is observed: in some cells nsP2 is found mostly in the nucleus whereas in others it is almost exclusively located in the cytoplasm. Most likely, this reflects the time-dependence of nuclear localization. Indeed, the fractionation of infected cells confirmed that at early stages (4 h) post infection the nuclear localization of nsP2 is prominent. In contrast, at later stages (12 h) post infection nsP2 is mostly located in the cytoplasm (Utt et al. 2015). The biological reasons, significance, and mechanisms responsible for this phenomenon are not known.

The ability of nsP2 to induce shutdown of cellular transcription, probably combined with other activities of the protein, makes it highly cytotoxic. The cytotoxic functions of nsP2 can be suppressed by different mutations introduced into the protein. Thus far no single mutation has been described that would completely eliminate the cytotoxic effects of CHIKV nsP2 and simultaneously allow virus replication. However, different groups have reported four combinations of mutations, each

allowing persistent growth of CHIKV replicon RNA in BHK-21 cells. For the ECSA genotype these are combinations of Pro718 to Gly substitution with 5 aa residue insertion between residues 647 and 648 of nsP2 (Pohjala et al. 2011) or with Glu117 to Lys substitution (Utt et al. 2015). For the WA genotype these are Pro718 to Ser substitution combined with double substitutions in the putative NLS region (Lys + Arg649 to Ala + Ala) or with substitution of Asp711 to Gly (Fros et al. 2013). For nsP2 of ECSA genotype, the above-mentioned mutations and their combinations compromise all the known enzymatic activities of nsP2 (Utt et al. 2015); similar data concerning the nsP2 of WA genotype are not available. Consistent with observations made for SINV and SFV (Tamm et al. 2008; Frolov et al. 1999), these mutations, in combination or alone, reduce the levels of viral RNA replication (Utt et al. 2015, 2016; Fros et al. 2013). The general tendency is that mutations having a bigger impact on cytotoxicity also have a more severe effect on RNA replication.

The effect of cytotoxicity reducing mutations on CHIKV replication and nuclear transport of nsP2 is one of the few occasions where data are available for viruses belonging to two different genotypes (ECSA and WA). The significant discrepancy of these results illustrates that information available for one CHIKV genotype does not necessarily apply for the other(s). Thus, Pro 718 to Ser mutation severely inhibits RNA synthesis of WA genotype replicons (Fros et al. 2013) and it has virtually no effect on the RNA synthesis of ECSA genotype replicons (Utt et al. 2015, 2016). Furthermore, in the WA genotype this mutation did not affect the nuclear localization of nsP2, whereas for the ECSA genotype virus the nuclear localization became more pronounced. The double substitution in the putative NLS region (Lys + Arg649 to Ala + Ala) of nsP2 of WA genotype virus prevents the nuclear transport of nsP2 (Fros et al. 2013) whereas insertion of 5 aa residues into the corresponding region of nsP2 of ECSA genotype also destroys the sequence of putative NLS but has absolutely no effect on the nuclear localization of nsP2 (Utt et al. 2015).

Infection of vertebrate cells with wild-type alphaviruses also results in rapid shut-off of cellular translation. Translation of viral genomic RNAs is also inhibited, but translation of subgenomic RNAs remains active (Strauss and Strauss 1994). The phenomenon of translation shut-off has been mostly studied using SINV as a model. It has been shown that inhibition of translation is achieved by both protein kinase R- (PKR-) dependent and PKR-independent mechanisms with the latter being the major pathway (Gorchakov et al. 2004). Importantly, it has been clearly demonstrated that transcriptional and translational shut-offs are independent events (Gorchakov et al. 2005). In order to achieve a noncytotoxic phenotype, the alphavirus replicons must lack the ability to inhibit both cellular transcription and translation. Thus, mutations in CHIKV nsP2 generating noncytotoxic replicons (Utt et al. 2015; Fros et al. 2013) eliminate both of these abilities. This would suggest that nsP2 is important for CHIKV-induced translational shut-off. To date, no nsP2 mutation, resulting in elimination of translational, but not transcriptional, shut-off has been reported for CHIKV. In contrast, such mutations have been described for SINV (Gorchakov et al. 2005; Mayuri et al. 2008). Thus far it is not known how nsP2 mediates translational shut-down, although pull-down experiments with SINV nsP2 have revealed that nsP2 interacts with several proteins involved in translation and

with almost the entire sets of ribosomal proteins composing both subunits (Atasheva et al. 2007). Similarly, association of nsP2 of New World alphavirus VEEV with the ribosomal protein RpS6 has been revealed. It has been demonstrated that VEEV infection results in rapid and dramatic decrease of RpS6 phosphorylation which may be important for alphavirus induced translational shut-off (Montgomery et al. 2006). However, as an alternative explanation, it may be that translational shut-off is mainly a host antiviral response, and the low levels of replication in the noncytotoxic mutants are unable to induce this reaction.

In addition to causing a general shut-off of cellular transcription and translation, CHIKV infection also specifically suppresses antiviral responses mediated by type I/type II interferons (IFN). Such a phenomenon is common for all alphaviruses; for Old World alphaviruses, it has been revealed that this is also caused by nsP2 (Breakwell et al. 2007; Frolova et al. 2002; Nikonov et al. 2013). Using transient expression of nsP2 and replicon vector of CHIKV WA genotype it has been shown that CHIKV is no exception to this rule, and its nsP2 is a potent inhibitor of IFN-induced JAK-STAT signaling. The Pro718 to Ser mutation in CHIKV-nsP2, when introduced into a replicon vector, significantly reduced JAK-STAT inhibition (Fros et al. 2010). However, because in transient expression experiments the same mutation failed to reduce the nuclear translocation of STAT1 (Fros et al. 2013), it is likely that the effect detected in the replicon experiment was mostly, if not exclusively, indirect (due to the reduced RNA replication and nsP2 synthesis). In contrast, nsP2 carrying a mutation in the putative NLS (Lys + Arg649 to Ala + Ala) completely lost the ability to inhibit the nuclear translocation of STAT1. Thus, regardless of the actual mechanism of its nuclear entry, the nuclear localization of nsP2 is strictly required for blocking of the JAK-STAT pathway (Fros et al. 2013). This conclusion is also supported by results obtained using ECSA genotype virus (Utt et al. unpublished). Thus, host shut-off-independent inhibition of IFN signaling is common for different CHIKV genotypes and is likely to have an important role in viral pathogenesis.

Interaction with other viral and host proteins. In replication complexes all the ns-proteins of alphaviruses interact with each other directly or indirectly. This has been confirmed by pull-down experiments performed using tagged nsP2, nsP3, or nsP4 (Atasheva et al. 2007; Cristea et al. 2006, 2010; Frolova et al. 2006). There are also internal domain–domain interactions within these large proteins as indicated above for nsP2 (Das et al. 2014b). There is convincing evidence showing a functional interaction between the N-terminal region of nsP2 and the N-terminal domains of nsP3 for SFV (Lulla et al. 2012). Finally, for SINV the 3D-structure of a polyprotein, representing the C-terminal part of nsP2 and the first two domains of nsP3, has been resolved. The revealed structure shows that nsP2 and nsP3 share an extensive interface with 3000 Å^2 of buried surface area. The interface between nsP2 and nsP3 is charged, with the nsP2 surface being mostly basic, whereas nsP3 is generally acidic. The significance of the interaction of these two proteins was highlighted by site-directed mutagenesis (Shin et al. 2012). Interestingly, the major aa residue affecting cytotoxicity (nsP2 Pro718) is located at the interface, and could thus affect the conformation of the polyprotein causing multiple functional alterations.

For CHIKV, the most complete study of viral–host protein interactions has been performed with a yeast two-hybrid system (Bourai et al. 2012). Using full-length nsP2 and seven different fragments of nsP2 as bait Bourai and colleagues identified in total 21 different hits; 15 of them were subsequently positively validated using biochemical assays. Importantly, using nsP2s of SFV and SINV in the same assay, 10 interactions were validated with all three viruses, and 6 more were validated with two out of three viruses. Hits validated for CHIKV included nuclear proteins (hnRNP-K, SRSF3), cytoskeleton components (VIM, TACC3, CEP55, KLC4), regulators of gene transcription (ASCC2, TRIM27, MRFAP1L1, EWSR1, IKZF1, ZBTB43), and host factors involved in protein degradation and/or autophagy (CALCOCO2/NDP52, UBQLN4, RCHY1, WWP1). It was proposed that CHIKV interacts through nsP2 with these cellular functions. However, it was observed that siRNA-mediated knockdown of most of these proteins has little effect on CHIKV replication. The exceptions from this were hnRNP-K and UBQLN4, as knockdown of their expression substantially inhibited CHIKV replication (Bourai et al. 2012).

Cellular processes other than the IFN response also affect CHIKV infection. For example, autophagy (a catabolic cellular process, which sequesters cytosolic components within double-membrane vesicles and targets them for degradation) has been shown to play different important roles in the outcome of CHIKV infection. Interestingly, autophagy plays an antiviral role in mouse cells where it delays caspase-dependent cell death (Joubert et al. 2012), but has a proviral role in CHIKV-infected human cells (Krejbich-Trotot et al. 2011). It was found that this difference can be explained by the fact that human autophagy receptor NDP52, but not its mouse orthologue, interacts with CHIKV nsP2 (and also with SFV and SINV nsP2). The interaction of nsP2 with human NDP52 can be detected by coimmunoprecipitation and yeast two-hybrid system. Furthermore, in infected human cells NDP52 localizes in the vicinity of CHIKV replicase complexes. It was proposed that NDP52–nsP2 interaction in the cytoplasm restricts the nuclear localization of highly cytotoxic nsP2 and delays cell death allowing prolonged replication. As mouse NDP52 does not interact with nsP2, autophagy is antiviral in mouse cells (Judith et al. 2013).

It has been proposed that alphaviruses are sensitive to the effects of the unfolded protein response (UPR), which is triggered by ER stress resulting from the expression of alphavirus glycoproteins (Barry et al. 2010). Therefore it is not surprising that CHIKV has developed a strategy to avoid UPR during infection. In cells infected with CHIKV, the splicing of XBP1 mRNA (a characteristic event in UPR) is incomplete and at a late stage of infection the cells become resistant to treatment with tunicamycin, a potent inducer of UPR. As transient expression of CHIKV glycoproteins triggers UPR, the ability to suppress UPR is associated with the nonstructural region. Indeed, it was also shown that transient expression of N-terminally tagged nsP2 (from CHIKV of WA genotype) is sufficient for UPR suppression and that mutations, known to suppress cytotoxic properties of nsP2, make the protein unable to interfere with UPR (Fros et al. 2015).

Antivirals targeting nsP2. The proteases of human immunodeficiency virus type 1 and hepatitis C virus have been successfully used as targets for antiviral compounds.

Therefore the highly multifunctional CHIKV nsP2, especially its protease region with known 3D structure, represents an attractive target for the design of antiviral compounds.

In their study, Bassetto and coworkers used the 3D structure of nsP2 protease, obtained by homology modeling, for virtual screening of a large library of commercially available compounds. The initial screen was followed by molecular docking and then by several rounds of screening on CHIKV-infected cells. One of the initial hits was shown to have an IC_{50} value of ~5 μM and some compounds, structurally related to it, had similar IC_{50} values. The most potent compound, resulting from this multistep screening process, had an IC_{50} value of ~3 μM and a selectivity index of 32 (Bassetto et al. 2013). In another study, carried out by Jadav and coworkers, series of arylalkylidene derivatives of 1,3-thiazolidin-4-one were synthesized and tested in a similar cell culture assay. In this study, the most active compound had an IC_{50} below 1 μM and molecular docking simulation suggested protease inhibition as a possible mode of action (Jadav et al. 2015). Recently, for the first time, a new panel of predicted nsP2 inhibitors was shown to inhibit both the protease activity of nsP2 as well as CHIKV replication in cell culture (Das et al. 2016).

An alternative cell-based assay is taking advantage of the ability of nsP2 to suppress cellular transcription. A screen of 3040 compounds led to identification of a single nontoxic natural compound that inhibited nsP2-mediated cytotoxicity and was also a weak inhibitor (IC_{50} = 31 μM) of CHIKV replication in cell culture. The mechanism of action of this compound and whether it targets nsP2 or a host component, involved in the development of nsP2-mediated cytotoxicity, is currently unknown (Lucas-Hourani et al. 2013).

nsP3

Alphavirus nsP3 can be divided into three different domains (Fig. 4). The N-terminal domain of ~160 aa is known as the macro domain, and the crystal structure of the CHIKV macro domain has been determined (Malet et al. 2009). The macro domains are named after macrohistones, unusual types of histones, which in addition to the canonical histone domain contain the macro domain. Macro domains are also found in other proteins, and the human genome expresses 11 proteins encoding 1–3 macro domains (Feijs et al. 2013). In RNA viruses, macro domains are expressed by

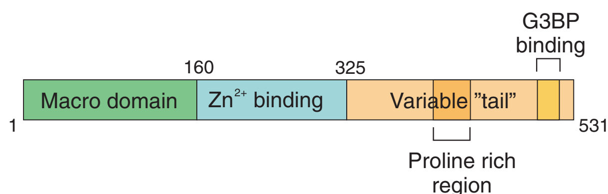

Fig. 4 The domain structure of nsP3, and the motifs important for association with host proteins

alphaviruses, rubella virus, hepatitis E virus, and the otherwise unrelated coronaviruses. The biochemical function of most macro domains is to bind mono-ADP-ribose (ADPr) or poly-ADP-ribose (PAR). Mono- or poly-ADP-ribosylation is a post-translational modification of many cellular proteins, and it is catalyzed by a large family of mono-ADP-ribosyltranferases and poly-ADP-ribose polymerases (Feijs et al. 2013; Li et al. 2016), some of which have been implicated in antiviral responses against alphaviruses (Atasheva et al. 2014; Bick et al. 2003). The current consensus is that many macro domains, including the virus-encoded ones, could act as mono-ADP-ribosyl hydrolases, removing the ADP-ribosyl marks on proteins (Feijs et al. 2013; Li et al. 2016). In vitro, the CHIKV macro domain can bind both mono- and poly-ADP-ribose, as well as RNA, and it can also hydrolyze the small substrate analogue ADP-ribose-1″-phosphate (Malet et al. 2009), suggesting that it might also release ADP-ribose bound to proteins. This is an exciting area where more biochemical and proteomic studies are required.

The macro domain is followed by another small domain, found thus far only in alphaviruses, which was defined when the crystal structure of a fragment of SINV replicase was solved. This domain binds a structural Zn^{2+} ion, coordinated by four conserved cysteines (Shin et al. 2012). Although this domain, like the macro domain, is essential for alphavirus replication, its precise functions remain to be determined. Both domains are involved as regulators of ns polyprotein processing, but they certainly have additional functions.

These two globular domains are followed by a “tail” region, which is variable in length and sequence between different alphaviruses. In CHIKV, the length of the tail is approximately 205 aa residues. The tail region is heavily phosphorylated on serines and threonines (Vihinen et al. 2001) and it is considered to be unstructured. As is typical for such protein segments, the tail can bind to multiple host proteins, and due to its variation, it could mediate several virus-specific and even cell-type-specific interactions with the host. The tail does tolerate large deletions and insertions, and in several viruses, including CHIKV, it has been used as an insertion site for marker proteins, including fluorescent proteins and *Renilla* luciferase (Pohjala et al. 2011; Kummerer et al. 2012). In spite of the variation, the tail also contains functional elements conserved in several or even all alphaviruses. A conserved proline-rich sequence stretch (PVAPPRRRR) in the tail binds the SH3-domain containing host proteins known as amphiphysin-1 and -2 in CHIKV, SFV, SINV, and most likely in all Old World alphaviruses (Neuvonen et al. 2011). For CHIKV, this interaction has been seen upon nsP3 transfection, but has not been verified in infected cells. For SFV and SINV, the amphiphysins are recruited to viral replication complexes (Neuvonen et al. 2011). They are proteins that can recognize or induce membrane curvature, and thus have been proposed to be structural components of the membranous replication spherules.

The nsP3 of CHIKV and other Old World alphaviruses also directly binds to G3BP1 and G3BP2, which are components of cellular stress granules, dynamic structures involved in arresting RNA translation. Recruitment of G3BPs by nsP3 prevents the formation of stress granules during infection. Instead, nsP3 recruits G3BPs to alternate, presumably virus-induced granules, which are devoid of many stress granule components (Fros et al. 2012; Panas et al. 2014). The binding is medi-

ated by two short, conserved repeat sequences close to the C-terminus of nsP3 (Panas et al. 2014). Therefore, in addition to the replication complexes, nsP3 is prominently localized in granular cytoplasmic structures (Neuvonen et al. 2011). G3BPs have a proviral function (presumably facilitating the switch from ns-polyprotein translation to RNA replication), as depletion of these proteins results in reduced CHIKV replication (Scholte et al. 2015; Kim et al. 2016; Schulte et al. 2016). Additionally, alphavirus nsP3 can interact with several other host proteins, but the significance of these interactions is as yet poorly understood.

Interestingly, recent results indicate that CHIKV nsP3 can be replaced by SFV nsP3 in a chimeric virus, which retains infectivity but becomes temperature-sensitive, only replicating well at 28°C (Merits, unpublished). This seems to reinforce the notion that the basic essential functions of the nsPs are conserved and can be replaced by homologues, but more specific (host) interactions may be altered in different alphaviruses and can be compromised in chimeras.

nsP4

nsP4 is the catalytic subunit of the alphavirus replicase, the RNA-dependent RNA polymerase (RdRp) (Rubach et al. 2009). Unlike many viral RdRps, the 3D structure of nsP4 remains unknown; however, based on sequence conservation and secondary structure prediction, it has the "right-hand" fold typical for RNA and DNA polymerases. It is clear that nsP4 is assisted in RNA synthesis by the other ns-proteins, which are probably also needed for the correct folding of nsP4. In addition to RdRp activity, nsP4 has terminal adenylyltransferase activity (Tomar et al. 2006), which is likely used for the synthesis of poly(A) tails of positive-strand RNAs in a template-independent fashion. In infected cells, nsP4 is the least abundant ns-protein. This is due to the in-frame terminator codon located upstream of nsP4 coding region (present in most alphaviruses and CHIKV isolates) and to the rapid degradation of individual nsP4 by the proteasome (de Groot et al. 1991). The degradation is induced by the N-terminal Tyr residue of nsP4 which has been shown to be crucial for the enzymatic activity of the protein (Shirako and Strauss 1998). When nsP4 is included in replicase complexes, it becomes stable. The N-terminus of nsP4 consists of 100 aa that could form a partially unstructured region, which precedes the canonical polymerase fold, but this region is essential for the functions of nsP4 (Rupp et al. 2011; Fig. 5).

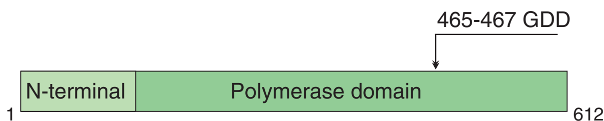

Fig. 5 The domain structure of nsP4, with the central polymerase motif Gly-Asp-Asp (GDD) marked

nsP4 is the most conserved protein of alphaviruses and it is very likely that the basic properties of CHIKV nsP4 are similar to the nsP4s of other alphaviruses. Thus far there are no reports of successful expression and purification of functional CHIKV nsP4 as a recombinant protein. Such attempts are hampered by instability of individual nsP4 in eukaryotic cells and/or by insolubility of recombinant nsP4 expressed using the bacterial system. Nevertheless there are convincing, though indirect, pieces of evidence that CHIKV nsP4 indeed acts as RdRp. Mutations in the canonical polymerase active site motif Gly-Asp-Asp (aa residues 465–467) completely abolish CHIKV RNA synthesis (Utt et al. 2016). Furthermore, application of chemical compounds affecting RNA synthesis results in mutations mapping to nsP4. Thus, a mutation associated with resistance to a broad-spectrum viral polymerase inhibitor Favipiravir maps to aa residue 291 of nsP4 (Lys291 to Arg; Delang et al. 2014). Selection of CHIKV mutants in the presence of ribavirin and 5-fluorouracil led to identification of Cys483 to Tyr mutation in nsP4. This mutation confers resistance to the mutagenic effects of these compounds by increasing the replication fidelity of CHIKV. However, this mutation also reduces the genetic diversity of viral progeny and negatively affects CHIKV fitness both in invertebrate and vertebrate hosts (Coffey et al. 2011). Conversely, it has been reported that changing of Cys483 to Ala, Gly, or Trp residue has an opposite effect and results in increased mutation frequency (Rozen-Gagnon et al. 2014).

As indicated above, alphavirus infection results in ER stress and may trigger UPR. It has been reported that during CHIKV infection it is antagonized by nsP2 (see above; Fros et al. 2015). Interestingly, a study by Rathore and coworkers revealed that nsP4 may also have a role in this process. It was observed that the splicing of XBP-1 mRNA is affected in CHIKV infection. Furthermore, it was noticed that compared to SINV, which induces the rapid phosphorylation of translation initiation factor eIF2α at Ser51, CHIKV possesses mechanism(s) that allow the avoidance of eIF2α phosphorylation. Transient overexpression of EGFP-nsP4 fusion protein was found to be sufficient to prevent eIF2α phosphorylation induced by subsequent tunicamycin treatment (Rathore et al. 2013). The mechanism behind this effect is unknown. Similarly, it remains unknown whether nsP4, expressed in natural CHIKV infection, has the same effect.

The N-terminal region of nsP4 has been shown to have functional linkage with all the other ns-proteins (Fata et al. 2002; Shirako et al. 2000; Rupp et al. 2011). In contrast, little information is available concerning the cellular proteins interacting with nsP4 in any alphavirus as such studies are hampered by the requirement of an aromatic N-terminal aa-residue, low abundance, and low stability of nsP4. Nevertheless, at least one important cellular interaction partner is known for CHIKV nsP4: heat shock protein 90 alpha subunit (HSP-90α). This interaction was first identified using coprecipitation with overexpressed EGFP-nsP4 protein and, importantly, then confirmed by reverse pull-downs using antibodies specific for HSP-90α. Finally, the functional significance of this interaction was confirmed by siRNA knock-down experiments. Based on these data it was proposed that the interaction between CHIKV

nsP4 and HSP-90α might be critical for the assembly of the viral replication complex (Rathore et al. 2014). In the same study HSP-90, more specifically HSP-90β, was shown to interact with CHIKV nsP3. Subsequently Das and coauthors reported that HSP90 affects the stability of nsP2 to a larger extent than it affects the stability of nsP3 or nsP4 (Das et al. 2014a). HSP90 was also coprecipitated with an antibody against nsP2 and vice versa. However, as in that study extracts of CHIKV-infected cells (rather than extracts of cells expressing individual nsP2) were used it remains possible that the interaction between nsP2 and HSP90 is mediated by nsP4 and/or nsP3.

Replication Complex

The RNA replication of all positive-strand RNA viruses takes place in association with cellular membranes (Paul and Bartenschlager 2013; Salonen et al. 2005). The viruses actively modify various membranes to build platforms and protective environments for efficient replication. The alphaviruses generate thousands of small membrane invaginations termed spherules (Spuul et al. 2010). It is thought that one spherule corresponds to one replication complex and each spherule would therefore contain one negative strand. The spherules arise concomitantly with virus RNA synthesis. They are very stable structures, and after being formed, they are active for several hours in producing multiple positive-strand genomic and subgenomic RNAs (Sawicki et al. 2006; Fig. 6). The spherule interior is always connected to the cytoplasm by a narrow neck, allowing the exit of RNAs and the entry of nucleotides. In mammalian cells, the alphavirus spherules are initially formed on the plasma membrane, and in some alphavirus-infected cells they are later endocytosed and found on the cytoplasmic surfaces of endosomes and large, vacuolated lysosomes, termed cytopathic vacuoles type I (Frolova et al. 2010; Spuul et al. 2010; Chen et al. 2013).

Many other viruses also give rise to similar replication spherules, although depending on the virus they have different localizations. For example, the well-characterized spherules of brome mosaic virus are located on endoplasmic reticulum membranes, and the nodavirus spherules reside on the mitochondrial outer membrane (Kopek et al. 2007; Schwartz et al. 2002). The formation of spherules is still poorly understood. For brome mosaic virus, it has been proposed that one of the viral replication proteins would form an internal coating or shell, giving structural support for the spherule (Schwartz et al. 2002). However, in the case of alphaviruses there is no good evidence for the presence of a shell-like structure, and different hypothetical models have been proposed (Kallio et al. 2013). Host proteins must be involved in the formation of spherules. In cells, transient structures resembling spherules are generated by the ESCRT proteins, which are involved in making the internal vesicles on multivesicular endosomal structures. Although ESCRTs may be involved in spherule formation in some viruses (Barajas et al. 2009), there is no evidence that alphaviruses utilize the ESCRTs as host factors. Interestingly, during alphavirus replication the size of the replicating RNAs determines the size of the spherules, whereas in some other viruses the spherule size appears to be fixed by the proteins involved (Kallio et al. 2013).

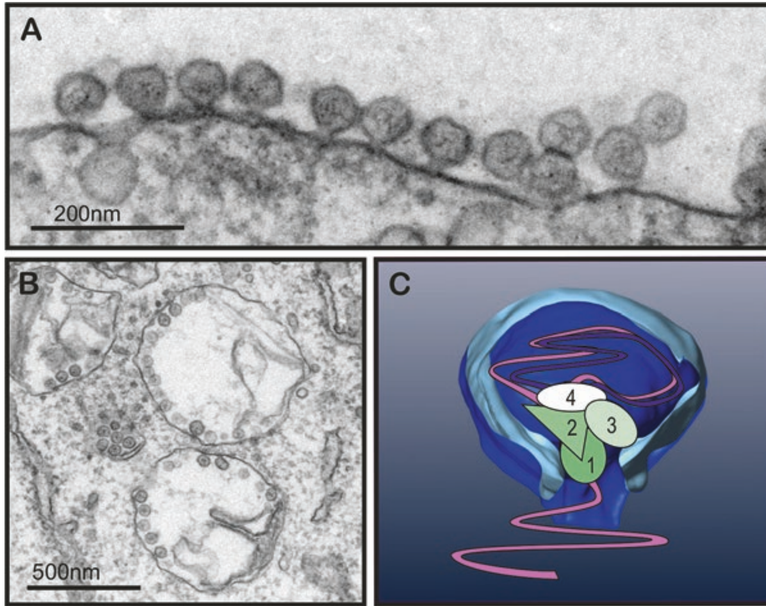

Fig. 6 (**a**, **b**) CHIKV replication spherules at the plasma membrane and on intracellular vacuoles in infected BHK cells, respectively. (**c**) Hypothetical model of a spherule, with the dsRNA replicative intermediate inside, and positive strand being released to the cytoplasm through the neck. The nsPs are not to scale, but are shown larger than predicted based on their molecular weight. Thus, multiple copies of some of the nsPs could be present. The location of the proteins (neck, or body of the spherules, or both) is also uncertain

As described above, many of the enzymatic and nonenzymatic activities of the nsPs have been characterized. However, it is still very poorly understood how they function together as a replication machine, coordinating at least the polymerase, helicase, and RNA capping activities. This issue is further complicated by the (likely) multimeric nature of the replicase and the restricted membranous environment in which it acts. Yet another unsolved facet is the interaction with and recruitment of the template to the replication complex. The sequences essential for alphavirus RNA replication are very well defined, and consist of the 5′ untranslated region (UTR) of the genome, a structured RNA element (replication enhancer) within the coding sequence for nsP1, and the 3′ end of the 3′ UTR followed by a poly-A stretch. The subgenomic promoter is only needed for the transcription of the subgenomic RNA but not for genome replication (Hellström et al. 2016). Although the core polymerase nsP4 needs to initiate RNA synthesis, the current results indicate that the presence of the other nsPs in the complex may be required for the recognition of at least some of the promoter elements (Li and Stollar 2004).

In conclusion, the methods to study the individual alphavirus nsPs both as recombinant proteins and in transfected and infected cells have been slowly developed over the last 30 years, and can now be applied to CHIKV. Many further methodological advances are needed in the coming years before the formation and functioning of the replication complex itself can be understood. A second area that still has many

unknown features, are the multiple virus–host interactions involved in antiviral responses, in the viral countermeasures, and in causing the pathogenic outcomes of CHIKV infection.

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Interaction of Chikungunya Virus with the Mosquito Vector

Konstantin Tsetsarkin, Lark L. Coffey, and Scott C. Weaver

Introduction

Chikungunya virus (CHIKV) is an arthropod-borne virus (arbovirus) transmitted by mosquito vectors. Aside from maternal-to-fetal infection during childbirth (Gerardin et al. 2008), all documented transmission to vertebrates occurs via the bite of an infected adult female mosquito (Coffey et al. 2014). As described below, the two distinct CHIKV transmission cycles, enzootic and urban, involve different mosquito vectors with important differences in ecology and behavior. However, the basic mechanisms of horizontal transmission among vertebrate hosts are essentially identical regardless of mosquito species. In addition to horizontal transmission, which results in human disease, vertical transmission from a female mosquito to her offspring has been reported in some studies (Agarwal et al. 2014) but discounted by others (Mourya 1987).

Certain aspects of CHIKV–vector interactions are relatively poorly understood and assumptions are made based on earlier studies of other alphaviruses and mosquito-borne arboviruses. However, the CHIKV genetic determinants of efficient infection of urban vectors, especially of adaptation for efficient transmission by *Aedes* (*Stegomyia*) *albopictus* as described below, are among the best understood of all arbovirus–vector interactions.

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Mosquito Biology

Mosquitoes are members of the insect family Diptera, and thus are holometabolous with complete metamorphosis between the aquatic larval and pupal stages and the terrestrial, flying adults (Clements 1992). All major CHIKV vectors are members of the genus *Aedes*, which are characterized by the laying of eggs singly on surfaces prone to flooding (Fig. 1). Thus, their populations of adults are unstable and generally respond to rainfall or water storage, ranging from treeholes for some of the enzootic vectors to artificial containers for the urban vectors. Most mosquito larvae are filter feeders and browsers on a variety of aquatic microorganisms, detritus, and biofilms, and undergo three molts to develop from the first instar that emerges from hatched eggs to the fourth instar that precedes the pupal state. Mosquito pupae do not feed and mainly serve as a site for metamorphosis between the larval and adult stages, where extensive tissue reorganizations occur.

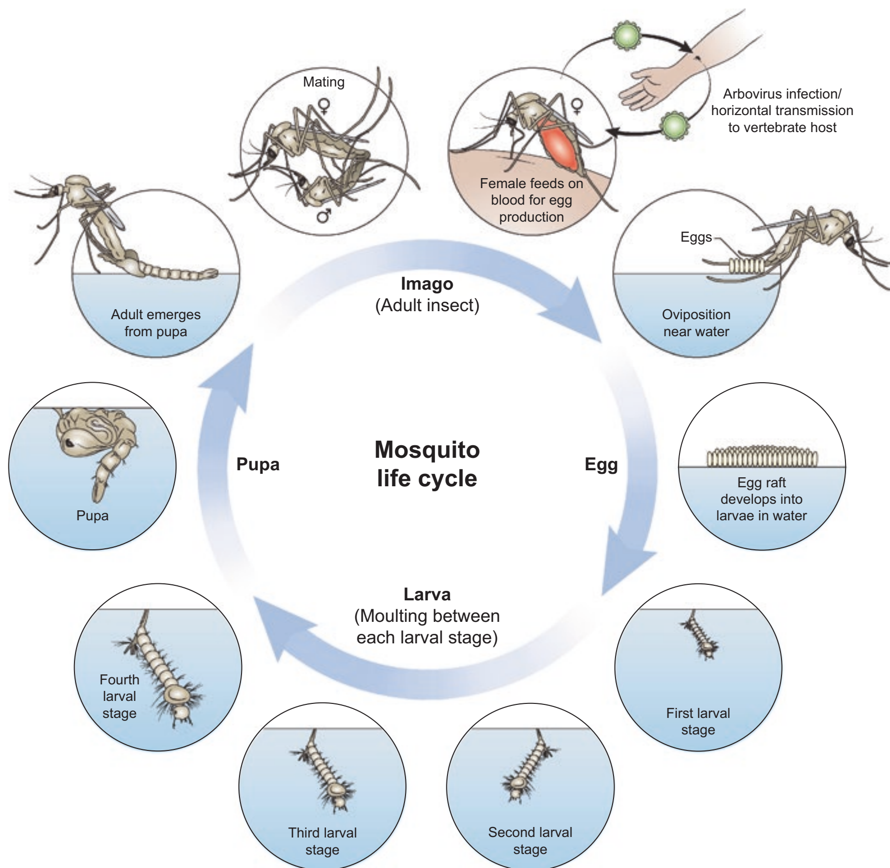

Fig. 1 Mosquito life cycle showing *Aedes* spp. eggs, 4 larval instars, pupal stage, and adult stage

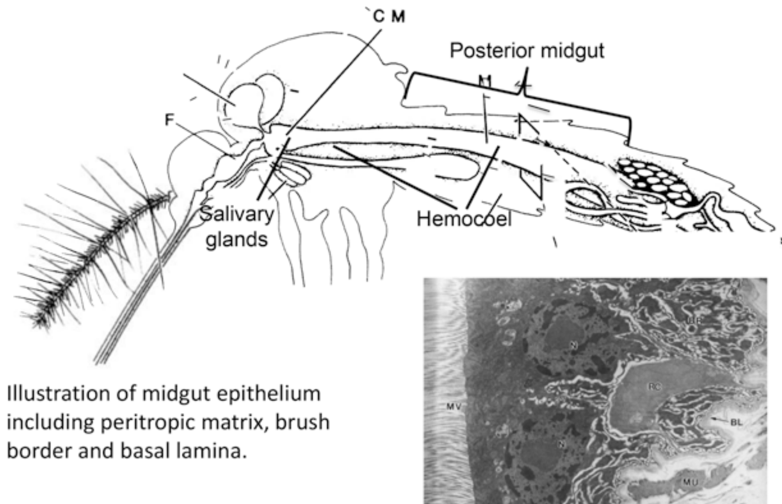

Fig. 2 Internal anatomy of the female mosquito showing the posterior midgut where blood digestion takes place, the hemocoel or open body cavity, and the salivary glands where replication must occur for transmission to take place upon subsequent blood feeding

Most adult mosquitoes principally feed on two kinds of fluids: plant secretions such as nectars containing sugars, and blood. Both males and females of most species feed on plant secretions, but adult females of most but not all species feed on blood, principally to derive the nutritional resources for egg production (Clements 1992). In a complex process involving the deposition of saliva to prevent coagulation and in some cases anesthetize the bite site to reduce vertebrate antimosquito responses, coupled with the localization and cannulation of venules and arterioles, mosquitoes generally ingest from 2–6 μL of blood. The entire volume is pumped into the highly distensible posterior midgut of the alimentary tract, where it remains for several days during digestion (Fig. 2). The midgut consists of a single layer of digestive epithelial cells along with regenerative and endocrine cells, surrounded by a basal lamina. The peritrophic matrix, a chitinous sac that is secreted by the midgut epithelial cells, forms within hours of feeding and encases the bloodmeal during digestion. This structure is excreted through the hindgut along with any undigested blood several days after feeding. Soon after ingestion, the erythrocytes clump in the center of the blood meal, with serum expressed to the periphery in contact with the epithelium. During midgut distention, blood digestion, and egg formation, neurohormonal signals release host-seeking and feeding behavior (Klowden 1996).

Arbovirus Infection of and Transmission by Mosquito Vectors

The process of mosquito infection and transmission of alphaviruses is not known to vary significantly among viruses or species, aside from transovarial transmission. Viremic blood passes into the posterior midgut during feeding, and comes into

direct contact with the epithelial cells during digestion. Concentration of serum as a result of blood clotting results in arbovirus concentration adjacent to the epithelium, enhancing infection (Weaver et al. 1991). Because the peritrophic matrix, which is believed structurally to exclude arboviruses, forms within hours of the bloodmeal, infection of the epithelial cells is presumed to occur mostly within a few hours of feeding (Houk et al. 1979). Replication of arboviruses is usually first detected within the posterior midgut 1–2 days after ingestion of viremic blood. However, dissemination into the hemocoel or open body cavity involves movement through the basal lamina, which is believed to be impervious in its static form (Fig. 2; Houk et al. 1981). Penetration may involve replication in tracheoblasts, which traverse the basal lamina, or possibly passage through the basal lamina during its remodeling. Once in the hemocoel, arboviruses have direct access to many organs and tissues including the salivary glands. Amplification is thought to involve the fat body, which occurs in many parts of the mosquito body and surrounds the salivary glands (Weaver et al. 1990). Like the midgut, the fat body and salivary glands are surrounded by basal laminae, and the mechanism of passage through this structure is not fully understood. Replication of alphaviruses in the salivary gland acinar cells leads to apical budding of nascent virions into the apical cavities, where saliva is stored prior to feeding. During the process of probing and feeding, virus is deposited into the vertebrate host, probably both extra- and intravascularly (Turell and Spielman 1992), to complete transmission.

CHIKV Vectors: Distribution, Ecology, Behavior, and Relative Importance for Viral Maintenance

Enzootic CHIKV Vectors in Africa

In Africa, CHIKV circulates in two distinct, yet sometimes overlapping transmission cycles. Similar to yellow fever virus, the enzootic (sylvatic) cycle of CHIKV involves nonhuman primates as amplifying host and a variety of primatophilic canopy-dwelling *Aedine* mosquitoes that include *A. furcifer-taylori*, *A. africanus*, *A. luteocephalus*, *A. neoafricanus*, and *A. cordellieri* (McCarthy et al. 1996; Weinbren et al. 1958; Jupp and McIntosh 1988, 1990; Diallo et al. 1999; Jupp and Kemp 1996; Fig. 3). Considering isolation frequencies as indicators of the importance for CHIKV transmission, it appears that *A. furcifer-taylori* is more important for CHIKV maintenance in western Africa (McIntosh et al. 1977; Diallo et al. 1999) whereas *A. africanus* is a more prevalent vector in central regions (Weinbren et al. 1958; McCrae et al. 1971; Jupp and McIntosh 1988; Tsetsarkin et al. 2011b). The ancestral, sylvatic, zoophilic African form of *A.* (*Stegomyia*) *aegypti* (*A. aegypti formosus*) has been found infected with CHIKV but is not considered an important vector (Diallo et al. 2012). Vector competence of some sylvatic mosquitoes for CHIKV has also been directly confirmed in laboratory experiments. The oral infectious dose 50 % (OID_{50}; defined as virus titer in the bloodmeal that is sufficient for infection of 50 % of mosquitoes) for CHIKV was below 6.2 $\log_{10}$ plaque-forming

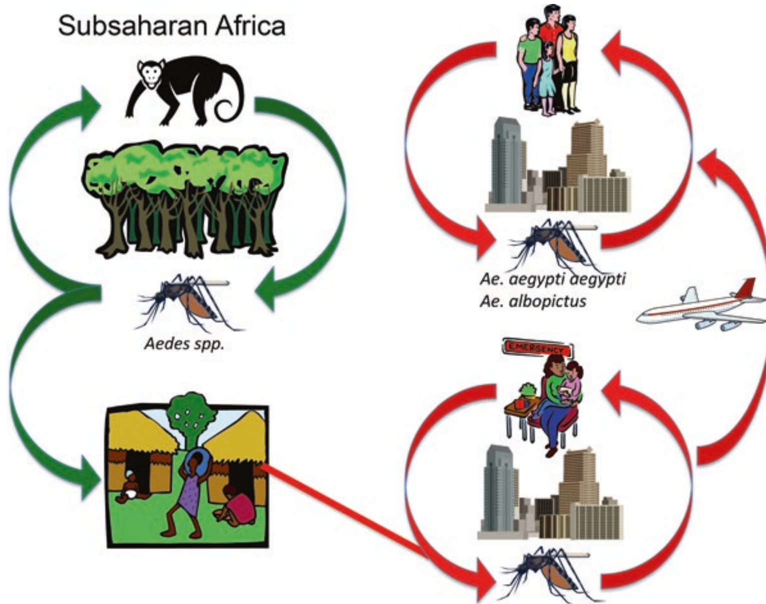

Fig. 3 Enzootic African and urban transmission cycles of chikungunya virus

units (PFU)/mL in *A. furcifer,* and infected mosquitoes were able to transmit at a rate of 25–32 %. Because experimentally infected vervet monkeys and baboons develop viremia up to 7–8 $\log_{10}$ PFU/mL, they are believed to be capable of serving as amplification hosts to sustain transmission of CHIKV (Jupp et al. 1981; Paterson and McIntosh 1964). Experimental transmission of CHIKV to rhesus macaques was also documented for *A. africanus* (Sempala and Kirya 1973).

The urban (endemic/epidemic) cycle is initiated after CHIKV introduction from African forests into peridomestic environments, where the peridomestic form of *A. aegypti* (*A. aegypti aegypti,* henceforth *A. aegypti*) and/or *A. albopictus* mosquitoes can support sustainable interhuman transmission. Transfer of CHIKV from enzootic to urban transmission cycles must involve the infection of people living near or working within forest habitats, followed by their movement to urban locations inhabited by *A. aegypti* and/or *A. albopictus* to initiate interhuman transmission. Of the enzootic vectors studied in Senegal, *A. furcifer*, which frequently enters villages and bites people, is the prime candidate for this bridge vector (Diallo et al. 2012).

Urban CHIKV Vectors in Africa

Historically, *A. aegypti* has been considered the principal urban CHIKV vector in Asia, and the only urban vector in Africa (Ross 1956; Diallo et al. 1999; Jupp and McIntosh 1988). During the course of its evolution in Africa, this peridomestic form of the species has acquired on several independent occasions anthropophilic traits

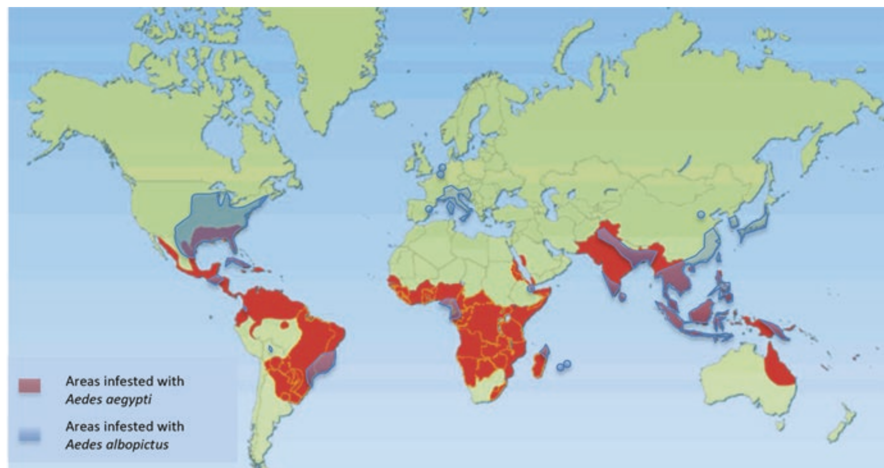

Fig. 4 Distribution of *A. aegypti* and *A. albopictus* worldwide

not found in the ancestral form *A. aegypti formosus*. This resulted in important behavioral changes such as the use of artificial water containers for larval habitats and a focus on humans for blood feeding (Powell and Tabachnick 2013; McClelland 1974; Tabachnick and Powell 1979; Failloux et al. 2002). The distribution of *A. aegypti formosus* versus *A. aegypti* in Africa has only been studied in a few locations, so the enzootic CHIKV foci near anthropophilic *A. aegypti* populations capable of initiating urban transmission are poorly understood. *A. aegypti* is readily infected with various strains of CHIKV under laboratory conditions. Depending on the virus and mosquito strains used, the OID_{50} values can range from 6.7 to 9.2 $\log_{10}$ PFU/mL, and CHIKV transmission rates to vertebrate hosts vary from 13–100 % (Jupp and McIntosh 1988; Ross 1956; Paul and Singh 1968; Gilotra and Shah 1967; McIntosh and Jupp 1970; Mangiafico 1971; Mourya 1987; Banerjee et al. 1988; Turell and Malinoski 1992; Vega-Rua et al. 2014; Coffey et al. 2014).

The decline in *A. aegypti* population in some parts of Africa due to mosquito eradication programs facilitated introduction and establishment of nonindigenous (to Africa) *A. albopictus* mosquitoes (Kamgang et al. 2011, 2013; Fig. 4). Facilitated by commercial shipping, these invasions occurred in a series of multiple introductions from poorly characterized tropical locations. *A. albopictus* was initially discovered in 1989 in South Africa, and subsequently was identified in many African countries including Gabon, Cameroon, Nigeria, the Central African Republic, and Equatorial Guinea (Paupy et al. 2009; Kamgang et al. 2011, 2013; Diallo et al. 2010). Invasion of *A. albopictus* does not result in displacement of the local populations of *A. aegypti,* as both species coexist sympatrically with shared larval habitats (Simard et al. 2005; Bagny et al. 2009). Early laboratory studies showed that *A. albopictus* is highly susceptible to CHIKV infection and can transmit virus to a vertebrate host (Singh and Pavri 1967; Paul and Singh 1968; Turell and Malinoski 1992). However, until 2006,

this species was considered only a secondary (after *A. aegypti*) CHIKV vector, mostly because of its less anthropophilic feeding preferences and less endophilic behavior compared to *A. aegypti*.

CHIKV Vectors in Asia

In contrast to Africa, in Asia only the urban CHIKV cycle has been described, with both *A. aegypti* and *A. albopictus* serving as primary vectors (Coffey et al. 2014). *A. albopictus* is native to Southeast Asia (Smith 1956; Fig. 4), whereas *A. aegypti* was repeatedly introduced to the region with commercial transport as early as the 1600s (Hawley 1988). This resulted in displacement of *A. albopictus* by *A. aegypti* in major Southeast Asian cities by the first half of the twentieth century. Therefore, CHIKV outbreaks in Asian cities are primarily vectored by *A. aegypti,* and virus transmission in the rural areas is supported mostly by *A. albopictus.* However, the discovery of high CHIKV seroprevalence among wild monkeys in the Philippines suggests the existence of sylvatic transmission there (Inoue et al. 2003). Field and viral genetic studies are needed to determine whether this represents temporary spillback from endemic transmission or a permanent enzootic cycle.

CHIKV Vectors in the Americas

Since its introduction into the Caribbean late in 2013, most transmission in that region has been assumed to occur via *A. aegypti* because *A. albopictus* is only found in the Cayman Islands, Dominican Republic, and Cuba among the Caribbean islands (Parola et al. 2006) (http://www.ecdc.europa.eu/en/healthtopics/vectors/mosquitoes/Pages/aedes-albopictus.aspx). In South, Central, and North America, *A. albopictus* is more widespread, indicating the potential for its involvement in CHIKV transmission (Fig. 4). However, previous genetic and vector competence studies indicate that the Asian lineage that was introduced is genetically constrained in its ability to adapt to *A. albopictus* due to an epistatic interaction with E1 amino acid 98 (see Section "Epistatic Interactions that Influence Penetrance of the E1-A226V Substitution and Impose Adaptive Constraints on CHIKV Evolution"; Tsetsarkin et al. 2011a).

In the United States, *A. aegypti* is normally only found in southern states where it can survive mild winters, and *A. albopictus* occurs farther north in the eastern half of the country (Eisen and Moore 2013) (http://www.cdc.gov/chikungunya/pdfs/CHIKV_VectorControl.pdf). Thus, if the Asian strain eventually adapts for more efficient transmission by *A. albopictus*, or if an Indian Ocean lineage (IOL) or east/central/south African lineage (ECSA) strain is independently introduced (http://www.scientificamerican.com/article/new-type-of-more-problematic-mosquito-borne-illness-detected-in-brazil/), CHIKV could expand its distribution northward (in the Northern hemisphere) and southward (in the Southern hemisphere) to threaten larger numbers of people in the Americas.

CHIKV Vectors in Europe

As in the Americas, *A. aegypti* is only present in a small area of Europe. However, *A. albopictus* has recently invaded southern areas of the continent in several countries (http://www.ecdc.europa.eu/en/healthtopics/vectors/vector-maps/Pages/VBORNET_maps.aspx). The much more widespread presence of *A. albopictus* is reflected in its involvement as the main vector in both European CHIK outbreaks: first in 2007 in northern Italy (Rezza et al. 2007), and then in 2010 in southern France (Grandadam et al. 2011); in both locations *A. albopictus* but not *A. aegypti* are present. Although the Italian outbreak involved an IOL strain with E1-226V, consistent with its adaptation for transmission by *A. albopictus*, the French isolates had E1-226A, which is usually associated with *A. aegypti* transmission. This finding underscores that *A. albopictus* is capable of transmitting both adapted and unadapted CHIKV strains.

Factors Affecting the Vector Competence of Mosquitoes for CHIKV

Vector Genetics

Variation in the susceptibility of *A. aegypti* and *A. albopictus* populations for CHIKV has been suspected for decades. Tesh et al. (1976) examined the susceptibility to infection among 16 geographic *A. albopictus* strains and found considerable variation as well as differences in mean CHIKV titers of up to 1000-fold within infected mosquitoes. Attempts to select for resistant or susceptible mosquito lines did not alter these susceptibility patterns. However, crosses between low and high susceptibility *A. albopictus* strains produced mosquito strains with intermediate infection rates suggesting a genetic component to susceptibility and virus production.

Another major study of vector susceptibility to CHIKV examined CHIKV transmission potential by *A. albopictus* in 6 geographic mosquito strains using 2 different virus strains and 2 temperatures of extrinsic incubation (20 or 28°C) (Zouache et al. 2014). Transmission potential was found to vary based on the combination of geographic mosquito strain, virus strain, and temperature. In the most extensive study published to date, Vega-Rua et al. (2014) assessed the susceptibility of 35 American *A. aegypti* and *A. albopictus* populations from 10 different countries to 3 different CHIKV genotypes and compared viral titers in mosquito saliva at two different times after oral infection. Infection and dissemination rates were similarly high for all mosquito populations and all 3 CHIKV strains, and up to 83 % and 97 % of *A. aegypti* and *A. albopictus* populations, respectively, were potentially transmission-competent (CHIKV present in heads, demonstrating dissemination into the hemocoel but not necessary shedding into saliva). However, considerable variation in saliva content was detected in both mosquito species, suggesting that replication in salivary glands varies to determine transmission efficiency. *A. albopictus* more efficiently transmitted the epidemic IOL strain CHIKV_0621, from La Réunion Island with E1-226V than

did *A. aegypti*, whereas the efficiency was reversed for the ECSA strain CHIKV_115 strain from La Réunion with E1-226A as well as the Asian genotype strain CHIKV_NC (Vega-Rua et al. 2014).

Molecular Antiviral Mechanisms in Mosquitoes (RNAi)

Inasmuch as mosquitoes, like all invertebrates, lack adaptive immune responses, their antiviral defense mostly relies on activation of innate immune mechanisms. Among them, the most significant for controlling replication of alphaviruses in mosquitoes is the small interfering RNA (siRNA) pathway (Myles et al. 2008; Ding 2010). This response is triggered when the ribonuclease Dicer2 recognizes and processes double-stranded (ds) RNAs, which occur during viral RNA replication. The resulting siRNAs of ~21 nucleotides interact with the RNA-induced silencing complex (RISC) to form a complex that degrades a complementary viral RNA (Sanchez-Vargas et al. 2009). The inhibitory role of the siRNA pathway was demonstrated for several members of the *Alphavirus* family, including CHIKV, in cell cultures and adult mosquitoes (Adelman et al. 2013; McFarlane et al. 2014; Campbell et al. 2008; Keene et al. 2004; Myles et al. 2008). Another class of regulatory molecules called piwiRNA-like virus-derived small RNAs are generated without involvement of Dicer2; however, similar to siRNA, they also appear to be produced from dsRNA intermediates (Morazzani et al. 2012). CHIKV infection of mosquito cells leads to production of piwiRNA-like virus-derived small RNAs, which modulate pathogenesis of CHIKV infection in mosquito cells (Morazzani et al. 2012). Moreover, knockdown of the piwiRNA pathway increases replication of the alphavirus Semliki Forest virus (SFV), suggesting that piRNA and siRNA pathways have synergistic effects in controlling viral infection of mosquito cells (Schnettler et al. 2013). There is no evidence that alphaviruses including CHIKV use a specific mechanism to inhibit siRNA or piwiRNA-like responses. It therefore was suggested that this response might have an overall beneficial effect on virus transmissibility by attenuating pathogenic effects associated with virus replication, which could prolong mosquito survival (Martin et al. 2010).

Finally, it was recently shown that other innate immune mechanisms such as JAK/STAT, IMD, or Toll pathways do not participate in inhibition of CHIKV replication in *A. aegypti*-derived Aag2 cells; however, the Toll signaling pathway is repressed in CHIKV-infected cells (McFarlane et al. 2014).

Role of Mosquito Microflora

Some populations of *A. albopictus* are naturally infected with *Wolbachia* (wAlbA and wAlbB), and these infections influence CHIKV replication (Mousson et al. 2010). In *Wolbachia*-infected mosquitoes, CHIKV infection results in a decline in *Wolbachia* density in the midgut and the salivary glands. Mosquitoes cleared of

Wolbachia infection show no change in survival or oviposition, nor in early CHIKV replication. However, infection appears to stabilize CHIKV replication levels (Mousson et al. 2010). Other studies have examined the effect of CHIKV infection on the *A. albopictus* gut microbiome (Zouache et al. 2012). Bacteria from the *Enterobacteriaceae* family increase in abundance with CHIKV infection, whereas other endosymbionts including *Wolbachia* and *Blattabacterium* decrease. These viral–microbiome interactions deserve further attention as a potential strategy to affect CHIKV transmission.

Other Extrinsic Factors Affecting CHIKV Transmission by Mosquitoes

In addition to the roles of intrinsic mosquito-specific factors, vector competence for CHIKV is also influenced by an array of external bioecological factors. For example, variation in ambient temperature has been shown to affect transmission efficiency of two CHIKV isolates by different geographic strains of *A. albopictus* (Zouache et al. 2014). It was also found that an increase in the larval rearing temperature of *A. albopictus* results in a decrease in adult mosquito susceptibility to CHIKV (Westbrook et al. 2010), and maintenance of *A. aegypti* at lower temperatures results in increased CHIKV susceptibility (Adelman et al. 2013). The inhibition of the RNAi response at lower temperatures has been proposed as a mechanism explaining the increase in mosquito susceptibility to CHIKV (Adelman et al. 2013).

Other environmental factors that may affect the susceptibility of mosquitoes to arboviruses include: insecticide exposure, availability of food sources, mosquito population density, and predation (Alto et al. 2005; Muturi and Alto 2011; Muturi et al. 2011a, b, 2012; Grimstad and Haramis 1984; Lounibos 2002; Rey et al. 2006; Pesko et al. 2009; Westbrook et al. 2010), although their impact on CHIKV vector competence remains to be studied.

Prospects for CHIKV Control Through Vector Manipulation

History of poor success in controlling dengue virus transmission via vector control (Gubler 1998) bodes poorly for CHIKV control. Traditionally, source reduction of containers that hold water exploited as larval habitats by *A. aegypti*, including backyard, nondegradable trash containers, has been a mainstay of control efforts. The periodic application of insecticides to containers containing larvae has also been used, but both of these methods have failed to control DENV and CHIKV vectors in a sustained manner due to the ability of *A. aegypti* and *A. albopictus* to exploit diverse larval habitats in urban and suburban regions (Corbel et al. 2013).

Because adult female *A. aegypti* often rest inside houses where traditional methods of outdoor adulticide spraying is not effective, indoor spraying with residual insecticides has shown the greatest promise for controlling this vector (Chadee 2013). *A. albopictus* tends to be more exophilic than *A. aegypti*. However, insecticide resistance threatens the success of even highly spatially targeted control approaches.

Newer strategies for the control of DENV and CHIKV transmission include the release of transgenic *A. aegypti* engineered to carry a late-acting lethal genetic system (Phuc et al. 2007). When adult male mosquitoes carrying this gene are released and mate with wild-type females, their progeny do not survive.

The artificial infection of CHIKV vectors with *Wolbachia* derived from *Drosophila melanogaster* has been evaluated as an approach to reduce transmission and human disease. In *A. aegypti* infected with the wMel strain of *Wolbachia,* CHIKV infection and dissemination rates are reduced compared to uninfected controls (van den Hurk et al. 2012). In *A. albopictus* artificially infected with the wMel strain, CHIKV dissemination to the saliva is inhibited with no detectable effects on fecundity, adult longevity, or male mating success (Blagrove et al. 2013). These results suggest that introducing wMel *Wolbachia* into natural *A. aegypti* and *A. albopictus* populations facilitated by their natural cytoplasmic incompatibility selection could be effective in reducing CHIKV transmission.

Role of Viral Genetic Factors in CHIKV–Vector Interactions

Historic Overview

The first evidence suggesting that viral genetics can influence CHIKV transmissibility by a mosquito vector came from the study described above comparing the susceptibility of various geographic strains of *A. albopictus* to two strains of CHIKV. A hundredfold difference in the OID_{50} values was detected between Asian and ECSA strains, which may reflect differences in laboratory passage history of the strains rather than true CHIKV lineage-specific variation (Tesh et al. 1976). Subsequently, significant variation in transmission potential (defined as time between ingestion of infectious blood and the time when the mosquito is capable of virus transmission) was detected among three Asian and one ECSA CHIKV strains in *A. aegypti* (Mourya et al. 1987), and in infectivity and dissemination among four West African CHIKV strains in *A. vittatus* and *A. aegypti* (Diagne et al. 2014). These observations, supported by similar phenomena documented for other members of the *Alphavirus* genus (Kramer and Scherer 1976; Green et al. 1980; Turell et al. 1999, 2003) suggested that fitness variations for infection of mosquitoes are not random fluctuations but reflect active adaptation. Rapid advances in molecular virology in the late twentieth century made possible detailed examination of the role of viral genetics in this process.

A. albopictus and Appearance of the CHIKV E1-A226V Substitution

It had been previously established that *A. albopictus* mosquitoes are susceptible to CHIKV infection and can transmit virus in laboratory settings with efficiency comparable to or greater than that of *A. aegypti* (Singh and Pavri 1967; Mangiafico 1971; Turell and Malinoski 1992). However, until 2005, this vector was believed to play only a minor role in CHIKV transmission, even though it was present in Asian regions endemic for CHIKV. The historically lesser role of *A. albopictus* in urban CHIKV transmission is assumed to result from the stronger anthropophily exhibited by *A. aegypti* (Table 1). In this context, the 2005–2008 CHIKV outbreak on Réunion Island was highly unusual because of the strong evidence incriminating *A. albopictus* as the primary vector, including: (1) the detection of viral RNA and virus isolation from field-collected females [Xavier de Lamballerie, cited in (Tsetsarkin et al. 2007; Delatte et al. 2008); and (2) the predominance of *A. albopictus* compared to *A. aegypti*, which had a very limited distribution on the island. Also, it was suggested that local *A. albopictus* populations might be more anthropophilic compared to mosquitoes of this species from other geographic locations (Reiter et al. 2006). A detailed temporal analyses of CHIKV genome sequences revealed an A to V substitution at position 226 of the E1 glycoprotein (E1-A226V; Fig. 5) in the majority of CHIKV strains isolated during the late phase of epidemic, but which was absent in all sequenced CHIKV strains isolated early during the Réunion outbreak or in the progenitor ECSA strains (Schuffenecker et al. 2006). It therefore was suggested that, in this location with few *A. aegypti* available for transmission, E1-A226V could have been selected as a result of direct adaptation of CHIKV for more efficient transmission by *A. albopictus*. This hypothesis was further supported in subsequent years during a series of similar outbreaks, all of which were associated with *A. albopictus* as the primary vector concurrent with the acquisition of the E1-A226V substitution. Thus, this mutation occurred convergently in independent CHIKV

Table 1 Characteristics of *A. aegypti* and *A. albopictus* important for chikungunya virus transmission

Trait	*A. aegypti*	*A. albopictus*
Distribution	Found throughout tropics and subtropics	Invaded many areas of tropics, subtropics, and temperate regions from Asia since 1985
Host preference for blood feeding	Feeds almost exclusively on humans	Feeds on humans as well as domestic and wild animals
Bloodmeals per gonotrophic cycle	Takes multiple bloodmeals within a gonotrophic cycle	Usually takes a single bloodmeal within a gonotrophic cycle
Larval habitats	Exploits artificial water containers near houses	Uses both artificial and natural larval habitats
Endophily	Adult females found mostly inside houses	Varied levels of anthropophily and endophily
Susceptibility to chikungunya virus infection	Moderate	Moderate to high

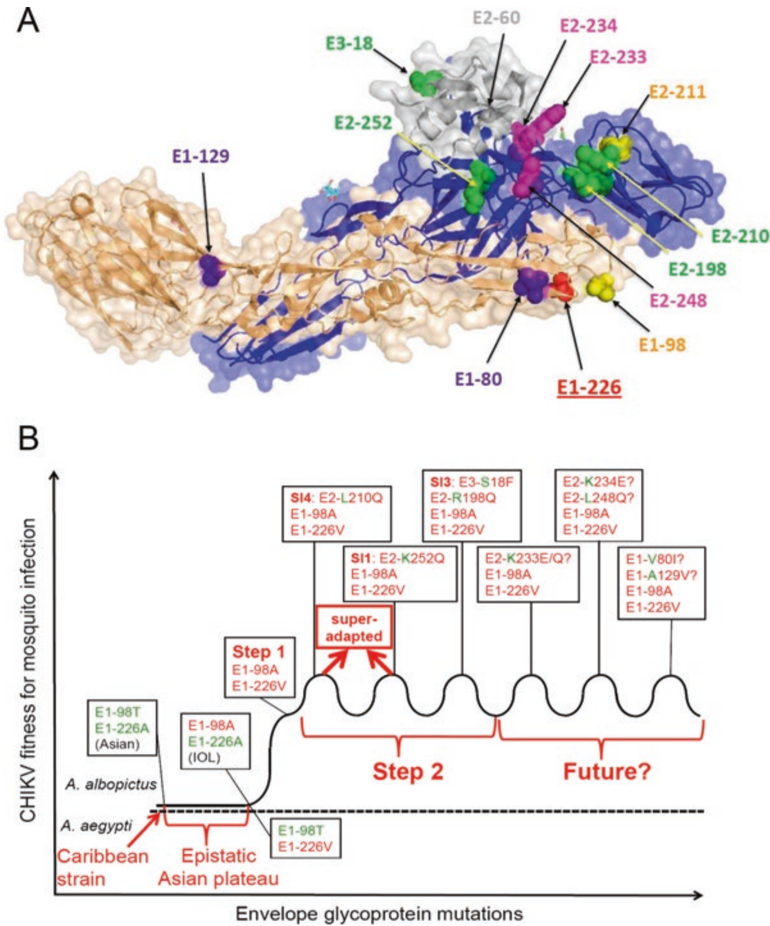

Fig. 5 (**a**) Map showing envelope glycoprotein substitutions that affect CHIKV fitness for transmission by *A. albopictus* based on a 3D model of E3/E2/E1 spike. The atomic structure of the CHIKV E3 (grey), E2 (blue), and E1 (gold) glycoprotein complex was generated based on [PDB ID:3N44 (Voss et al. 2010)]. The position of the first-step *A. albopictus*-adaptive E1-A226V substitution is indicated by the red sphere. Positions of substitutions that epistatically control penetrance of the first-step E1-A226V substitution are indicated by yellow spheres. The position of second-step *A. albopictus*-adaptive substitutions E2-L210Q, E2-R198Q/E3-S18F, and E2-K252Q are indicated by green spheres. The position of artificial substitutions (never reported in natural CHIKV isolates) that in laboratory experiments increased CHIKV transmissibility by *A. albopictus* are indicated by magenta and violet spheres. The grey sphere shows the position of a nonspecific determinant of CHIKV attenuation in *A. albopictus* and *A. aegypti.* (**b**) Simplified fitness landscape for a multistep process of host switching by CHIKV from *A. aegypti* to *A. albopictus* shows the existence of multiple independent second-step adaptive peaks available after the initial adaptive E1-A226V substitution (Tsetsarkin et al. 2014). Green indicates ancestral amino acids and red indicates derived, *A. albopictus*-adaptive. The Asian plateau represents the inability of Asian strains, including those introduced into the Caribbean in late 2013, to reach the first-step adaptive peak owing to its dependence on the epistatic E1-A226V/E1-98T interaction. Fitness for *A. aegypti* infection is not greatly affected by any of the *A. albopictus*-adaptive substitutions. At the right are potential future substitutions identified by prospective studies (Tsetsarkin et al. 2014; Stapleford et al. 2014)

lineages on at least 5 more occasions (Weaver 2014; de Lamballerie et al. 2008): in Kerala, India in 2007 (Dash et al. 2007; Arankalle et al. 2007; Cherian et al. 2009) and 2009 (Niyas et al. 2010), twice in Sri Lanka or in strains imported into Sri Lanka from India in 2008 (Hapuarachchi et al. 2010), and in 2007 in Gabon (Vazeille et al. 2008; Pages et al. 2009).

Effect of E1-A226V on CHIKV Fitness

The role of the E1-A226V substitution on CHIKV fitness in *A. albopictus* strains from several locations was directly evaluated using reverse-genetics experiments as well as natural CHIKV isolates. Using green fluorescent protein (GFP)-expressing viruses, it was shown that E1-A226V results in ~50–100-fold increase in CHIKV infectivity (Tsetsarkin et al. 2007, 2009, 2011a; Tsetsarkin and Weaver 2011; Table 2) and leads to more efficient viral dissemination to the salivary glands of *A. albopictus* (Tsetsarkin et al. 2007). Similarly, natural CHIKV strains from the Réunion outbreak with the E1-226V residue required ~50-fold lower titers in bloodmeals to achieve the same rate of dissemination into the mosquito head compared to strains with E1-226A, and were associated with significantly higher viral RNA loads in the mosquito midgut (Vazeille et al. 2007). Moreover, CHIKV with E1-226V was more efficiently transmitted to newborn mice compared to the E1-226A variant by orally infected *A. albopictus* (Tsetsarkin et al. 2007). Interestingly, in contrast to *A. albopictus,* the E1-A226V substitution has a neutral or slightly deleterious effect on CHIKV fitness in the prior (Chretien et al. 2007) or "donor" vector, *A. aegypti,* and in a mouse model of vertebrate infection. These findings further support the hypothesis that the E1-A226V substitution was acquired as a result of selection only by *A. albopictus* (Arias-Goeta et al. 2013; Tsetsarkin et al. 2007). As in previous studies of the adaptation of another alphavirus, Venezuelan equine encephalitis virus (VEEV), to a new mosquito vector (Deardorff and Weaver 2010; Brault et al. 2004), the lack of a major fitness cost for infection of the donor host (*A. aegypti*) in the adaptation of CHIKV to a new vector challenges the fitness trade-off hypothesis for host-specific adaptations. This hypothesis predicts that optimal adaptation to the donor host over long periods of evolution will be compromised when virus switches to a new host with a different infection and replication niche.

Table 2 Five natural *A. albopictus*-adaptive mutations found in Indian Ocean lineage strains

Sublineage	Protein	Substitution	Approximate Fitness increase
Multiple	E1	A226V	~50–100-fold
SL1	E2	K252Q	~6–8-fold
SL3B	E2/E3	R198Q/S18F (synergistic)	~6–8-fold
SL4	E2	L210Q	~4–6-fold

Nomenclature of sublineages is based on Tsetsarkin et al. (2014)

Mechanism of Enhanced A. albopictus Infection Mediated by the E1-A226V Substitution

Subsequent analyses of the E1-A226V substitution revealed that it does not affect CHIKV's ability to infect and replicate in the *A. albopictus*-derived C6/36 cells (Tsetsarkin et al. 2007, 2011c) and does not affect the virus' ability to be transmitted by intrathoracically injected *albopictus* (Arias-Goeta et al. 2013; Tsetsarkin 2011). Considering that the midgut is not required for virus replication and dissemination following intrathoracic infection, it was concluded that E1-A226V substitution provides a selective advantage at an infection stage preceding CHIKV dissemination from midgut into the hemocoel. To determine which step of infection within the midgut is the most affected by E1-A226V, CHIKV replicons expressing either GFP of cherry fluorescent protein (CFP) were packaged into virus-like particles (VLPs) using helper RNA encoding structural genes with the 226V or 226A residue in the E1 glycoprotein. VLPs containing replicons are only capable of a single round of infection and cannot spread to neighboring cells due to their lack of structural protein genes. This allows investigation of the effect of mutations of interest on the initial step of midgut epithelial cell infection following an infectious bloodmeal. The VLPs packaged using a helper encoding the 226V residue in the E1 glycoprotein are ~43 times more infectious to midgut epithelial cells of *A. albopictus*, compared to VLPs with the E1-226A residue (Tsetsarkin and Weaver 2011). Considering similar, ~50-fold difference in ability to infect and disseminate in *A. albopictus* between E1-226A and E1-226V-expressing infectious viruses (Tsetsarkin et al. 2007; Vazeille et al. 2007; Table 2), it appears that the increase in CHIKV infectivity for midgut epithelial cells associated with E1-A226V mutation is the primary mechanism of enhanced viral transmission and evolutionary success of E1-226V viruses in epidemics vectored by *A. albopictus.*

In the alphavirus virion, the E1 glycoprotein is mostly shielded from the surface of spikes by the E2 protein (Fields and Kielian 2013; Vaney et al. 2013; Voss et al. 2010), which includes the B domain thought to interact with cellular receptors (Fig. 5). This strongly suggests that E1-A226V does not directly affect receptor binding. Before the discovery of E1-A226V in CHIKV, a proline-to-serine substitution at the same position of the E1 protein of SFV (closely related to CHIKV) was shown to modulate the cholesterol dependency for viral entry into and exit from insect cells (Vashishtha et al. 1998). E1-226 is located at the tip of the ij loop of the E1 protein (Roussel et al. 2006; Voss et al. 2010) in close proximity to the fusion loop, and is engaged in close contact with target endosomal membranes during the viral fusion process. It was postulated that presence or absence of cholesterol in target membranes is "sensed" by E1-226, thus modulating the kinetics of the E1 conformational changes via interaction with the proline at E1-86, located at the base of fusion loop (Gibbons et al. 2000; Fig. 5). Because mosquitoes, like all insects, are cholesterol auxotrophs that acquire cholesterol needed for reproduction and development through dietary sources (Clayton 1964; Canavoso et al. 2001), mutations that influence alphavirus dependence on cholesterol may affect fitness for mosquito infection. Indeed, the E1-P226A substitution in SFV results in more efficient virus replication in *A. albopictus* infected intrathoracically as compared to the parental virus (Ahn et al. 1999).

Experiments using standard and cholesterol-depleted C6/36 cells demonstrated that, in addition to the effect on CHIKV fitness in *A. albopictus,* the E1-A226V substitution also makes CHIKV dependent on cholesterol for infectivity and replication in insect-derived cell cultures (Tsetsarkin et al. 2007; Tsetsarkin and Weaver 2011). However, genetic analysis of CHIKV mutants that vary in their sensitivity to cholesterol failed to support a mechanistic correlation between cholesterol dependency and increased *A. albopictus* infectivity. Moreover the E1-A226V substitution also results in increased pH-dependence of the CHIKV fusion reaction. However, subsequent analyses showed that pH-dependence also does not necessarily correlate with mosquito infectivity. Overall, these results suggest that E1-A226V substitution independently affects multiple functions of the virus, by acting at different steps of the CHIKV replication cycle (Tsetsarkin et al. 2011c).

Epistatic Interactions That Influence Penetrance of the E1-A226V Substitution and Impose Adaptive Constrains on CHIKV Evolution

The discovery that a single E1-A226V substitution, which was selected convergently on at least 6 independent occasions from 2006 to 2009, dramatically increases the epidemic potential of CHIKV in regions infested with *A. albopictus* (Weaver 2014; de Lamballerie et al. 2008), was largely unexpected. Considering that the geographic ranges of *A. albopictus* and CHIKV have overlapped for at least 60 years in southeast Asia since the 1950s (the latest date estimate for evolution of the Asian strain), and since the 1980s in Africa after the introduction of *A. albopictus* (Paupy et al. 2009; Gratz 2004), the question arises why the same E1-A226V mutation had not been observed during earlier CHIKV outbreaks.

The hypothesis first proposed for the lack of prior CHIKV adaptation to *A. albopictus* was that not all CHIKV strains/lineages are equally susceptible to the effects of the E1-A226V substitution. In other words, different CHIKV strains might have particular genetic signatures that limit penetrance of the E1-A226V substitution, thus restricting their ability to adapt to a new host. Although initial studies investigating the most phylogenetically distant strains (West African vs. IOL) of CHIKV showed no difference with respect to penetrance of the E1-A226V substitution (Tsetsarkin et al. 2007), further investigations revealed a more complex strain dependence, supporting a lineage-specific nature of CHIKV adaptation to *A. albopictus*.

Adaptive Constraints in Africa

Alphavirus infectious cDNA clones are plasmids containing the complete viral genome in cDNA form. They can be readily manipulated genetically, then transcribed and RNA electroporated into cells to rescue a genetically defined virus population. Introduction of the E1-A226V mutation into an infectious clone of the Ag41855 CHIKV strain, which is closely related to IOL strains, revealed no

significant difference in virus infectivity for *A. albopictus.* Using chimeric viruses constructed with Ag41855 and LR2006 OPY1 (a member of the IOL) CHIKV strains, followed by analysis of point mutations, two critical positions affecting mosquito infectivity of the Ag41855 genome were identified, both located in the E2 gene (Tsetsarkin et al. 2009; Fig. 5). The E2-60G residue was associated with ~10-fold decrease in CHIKV infectivity for both *A. albopictus* and *A. aegypti*, and only moderately modulated the penetrance of the E1-A226V mutation in *A. albopictus*. In contrast, the second Ag41855 residue, E2-211I, resulted in a ~50-fold reduction in CHIKV infectivity compared to E2-211T, but only for *A. albopictus* mosquitoes and only for CHIKV genomes that contained E1-226V. A phylogenetic analysis revealed high prevalence of E2-211I among ECSA strains, including those ancestral to the IOL. It is therefore conceivable that E2-211I residue may constrain the ability of some ECSA strains to utilize *A. albopictus* efficiently as an epidemic vector, thus limiting the potential for CHIKV emergence in some parts of Africa. The prevalence of E2-211I among ECSA strains may reflect its role in CHIKV adaptation to an enzootic sylvatic vector or nonhuman primate host, a hypothesis requiring further investigation. Alternatively, it is possible that E2-211I does not have any impact on fitness of CHIKV in the enzootic cycles and is simply an ancestral form.

The E2-I211T substitution, which does not interfere with penetrance of the E1-226V residue, was acquired by early IOL strains in 2004 in Kenya at the beginning of the 2004–2006 expansion into the Indian Ocean basin (Kariuki Njenga et al. 2008). Currently all strains that belong to IOL, as well as West African and Asian clades of CHIKV, have threonine residue at position E2-211. However, a possible selective advantage conferred by the E2-I211T substitution remains to be determined.

Adaptive Constraints in Asia

An even more striking example of how epistatic constraints can influence outcome of viral evolution and disease emergence was observed for the Asian CHIKV lineage, which evolved from the ECSA lineage, and has circulated in regions native to *A. albopictus* mosquitoes in Southeast Asia since at least 1958 (Volk et al. 2010). Even though all Asian strains possess the E2-211T residue, similar to the Ag41855 strain discussed above, introduction of the E1-A226V mutation into infectious clones derived from two different Asian strains of CHIKV does not increase their infectivity for *A. albopictus.* However, in contrast to ECSA strains, the inability of the Asian isolates to respond to E1-A226V was associated not with E2 but with a single residue 98T in the E1 glycoprotein (Fig. 5). The E1-98T is found in all endemic Asian strains sequenced to date, including those circulating in the Americas. Similar to E2-211I, introduction of the E1-A98T mutation into IOL strains almost completely blocks penetrance of E1-A226V. However, the introduction of the E1-T98A substitution into Asian isolates that have been mutated to E1-A226V leads to a ~100-fold increase in CHIKV infectivity for *A. albopictus.* The E1-98T has not been detected in any CHIKV strains except those in the Asian

lineage, suggesting that it may have become established as the result of a founder effect. This hypothesis is supported by the fact that this residue has no detectable effect on CHIKV fitness in the primarily historic Asian vector, *A. aegypti,* nor in small animal models of human infection. It is plausible that the inability of Asian CHIKV strains to adapt to *A. albopictus* left the potential human–*A. albopictus* niche unoccupied in Southeast Asia, thus enabling the rapid establishment of the *A. albopictus*-adapted IOL CHIKV strains in the region since 2006 (Tsetsarkin et al. 2011a).

A molecular explanation for the observed effect of mutations at E1-98 on penetrance of E1-A226V is that E1-98 can modulate flexibility of the fusion loop by interacting with proline at position E1-86, located at the base of the loop. The E1-98T may therefore override signals from residues at position E1-226, which interacts with E1-86P located on the base of the fusion loop. This could prevent fusion triggering in a specific environment of endosomal compartments of *A. albopictus* midgut cells during virus entry, thereby rendering CHIKV less infectious for this mosquito.

Second-Step Mutations in the E2 Glycoprotein Gene

Since discovery of the E1-A226V substitution, further research has shown that this was just the initial step in a complex process of CHIKV vector switching. A phylogenetic study of 2006–2009 CHIKV strains in the Indian state of Kerala identified that, in addition to the E1-A226V substitution, strains collected during 2009 acquired a novel E2-L210Q substitution (Niyas et al. 2010). The proximity of this mutation to the previously identified determinant of E1-A226V penetrance in *A. albopictus,* which is located at position E2-211 (Tsetsarkin et al. 2009; Fig. 5), prompted reverse-genetics investigations. It was shown that introduction of E2-L210Q in a CHIKV backbone that contained E1-226V resulted in a 4–6-fold increase in disseminated infection of *A. albopictus* (Table 2). Similar to the effect of the E1-A226V substitution, E2-L210Q primarily acts at the level of initial CHIKV infectivity for midgut epithelial cells, and has no effect on fitness for *A. aegypti* or human-derived cell lines (Tsetsarkin and Weaver 2011).

A subsequent phylogenetic analysis of CHIKV sublineages that had acquired the E1-A226V substitution revealed that this mutation is commonly followed by second-step substitutions in the E2 glycoprotein. In addition to 2009 strains from Kerala, two of three CHIKV sublineages examined contained additional *A. albopictus*-adaptive mutations. The E2-K252Q substitution (Fig. 5) first appeared in Kerala in 2007 (in a lineage independent from 2009 strains), which subsequently dispersed into numerous countries of Southeast Asia. The second sublineage was identified only in 2008 during a Sri Lankan outbreak (Hapuarachchi et al. 2010), and contained synergistic *A. albopictus*-adaptive mutations E3-S18F and E2-R198Q (Fig. 5); these residues must be expressed simultaneously in order to provide a 6–8-fold increase in CHIKV infectivity for *A. albopictus* (Tsetsarkin et al. 2014; Table 2). The ability of CHIKV to utilize various sets of adaptive mutations to achieve similar

selective advantages in a new host (ie, to occupy multiple adaptive peaks of relatively equal fitness) enabled the rapid lineage diversification that was observed in nature.

In addition to involving glutamine, all second-step *A. albopictus*-adaptive mutations share a common effector mechanism, involving selective 5–8-fold enhancement of initial CHIKV infection/replication in midgut epithelial cells of *A. albopictus.* All second-step mutations also map to the acid sensitive region (ASR) of the E2 protein that regulates low pH-induced E2-E1 heterodimer dissociation in the endosomal compartment. This pattern led to the hypothesis that, instead of affecting CHIKV–receptor interactions, these substitutions alter the mechanism of CHIKV entry, inducing fusion reaction in the early rather than the late endosome (Tsetsarkin et al. 2014).

CHIKV Population Genetics During Vector Infection

The repeated emergence of CHIKV E1-A226V since 2005 and laboratory competence studies that show E1-226V increases transmission by *A. albopictus* support a strong selective advantage in *A. albopictus*-inhabited regions for this mutation. Given the diffuse geographic distribution of *A. albopictus* including in CHIKV-endemic areas, questions arose as to why E1-226V had not been observed prior to 2005 outbreaks. One possibility is that the mutation was present in older isolates but because genomic sequencing was not standard in pre-2005 epidemics, it was not discovered. Another possibility is that it was generated, as are other random viral mutations produced by the errorprone viral polymerase, but that in the absence of *A. albopictus*, the mutation was not positively selected to dominate the mixed population of viral RNAs. Given conventional sequencing, a minority SNP would have been overlooked inasmuch as Sanger sequencing only measures dominant nucleotides. CHIKV outbreaks in Africa prior to 2005 typically used *A. aegypti* as the urban vector, and E1-226V may not have been positively selected in that vector; however, older outbreaks in Asia probably used *A. albopictus* as a secondary vector, therefore the conditions were probably permissive to promote the appearance of the mutation. A simple vector competence experiment using *A. aegypti* and *A. albopictus* that ingested CHIKV E1-226A, the ancestral genotype that initiated the 2005 ECSA IOL outbreak in Réunion Island, confirmed that a single passage through *A. albopictus* (but not *A. aegypti*) was sufficient to produce the E1-226V variant (Fig. 6). In that study, although E1-226V was observed on only a small minority of RNAs in the population of viral genomes in the mosquito hemocoel, it was the dominant transmitted variant in saliva samples. The fact that this epidemiologically important mutation appeared in a single mosquito passage in the laboratory and then arose to dominate the population of viral RNAs further complicates understanding of the origins of this mutation that had not been seen before 2005 despite CHIKV activity in *A. albopictus*-infested regions for decades or longer. Specifically, how could it be identified through such a simple experiment involving relatively little virus replication as occurs in a single mosquito? Although these questions

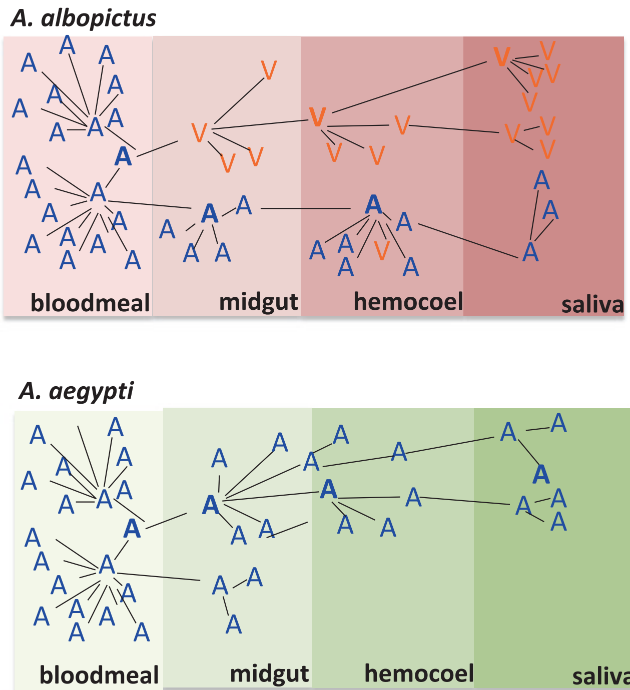

Fig. 6 CHIKV E1-226 population infection dynamics in *A. albopictus* and *A. aegypti*. Colored rectangles denote different compartments in mosquito infection and letters represent individual CHIKV genomes with the amino acid at E-1226 noted. Lines between letters are replication cycles that could mutate E1-226. Data from studies tracking virus populations (Stapleford et al. 2014) and studies that bypassed the midgut (Tsetsarkin et al. 2007) together show that E1-226V arises in *A. albopictus* presented infectious bloodmeals that contain only E1-226A, and that the mutation enhances CHIKV escape into the hemocoel. At transmission in saliva the mutation is expressed in most viral RNAs, affecting a consensus change, represented in bold. These infection dynamics are specific to *A. albopictus*; a similar pattern was not seen for *A. aegypti*

remain unanswered, these observations support the idea that the E1-226V mutation can circulate as a subdominant minority in a CHIKV population but is only fixed on all viral RNAs after positive selection in the right vector hosts.

The idea that CHIKV replication in vertebrate and invertebrate hosts produces mixed populations of viral RNAs that are highly similar but not identical is supported by in vitro and in vivo studies. However, the relative roles of individual RNA genomes, except for the specific E1 and E2 mutations described in the above sections of this chapter, and the impact of viral genetic diversity on infection, dissemination, or transmission patterns in vectors are largely unknown. Studies in *A. albopictus* and monkey kidney cells show that the genetic diversity (mutation frequency) and genetic distance (number of mutations by which each CHIKV RNA differs from the consensus [average] sequence) increase after both serial and alternating passage. The greatest increases in adaptability occurred in serially passaged CHIKV populations, suggesting that artificially removing vertebrate hosts (or conversely, invertebrate

hosts) from natural alternating transmission infections allows CHIKV to explore sequence space more expansively, resulting in mutations that confer fitness advantages (Coffey and Vignuzzi 2011). In vivo studies experimentally restricted diversity in CHIKV populations by employing a high-fidelity variant with a mutation at position 483 in the polymerase gene (NSP4-C483Y). Compared to wild-type CHIKV from Réunion Island, the high-fidelity variant produces ≈30 % fewer mutations, resulting in populations of viral RNAs that are less genetically diverse. Restricted diversity limits fitness of CHIKV in mosquitoes where high-fidelity CHIKV produces ≈10-fold lower infection and dissemination titers than wild-type in *A. aegypti* and *A. albopictus*, but does not affect transmission rates or doses delivered to vertebrates (Coffey et al. 2011). Supplementary studies of low-fidelity variants generated by mutating the amino acid at 483 in the polymerase, or the complementary position in Sindbis virus, showed that possessing hypermutated populations also compromises fitness in mosquito cells, and that this effect is due to deficits in viral RNA production. These patterns were not confirmed in *A. albopictus* or *A. aegypti* mosquitoes, probably because the mutant amino acids conferring low fidelity reverted to wild-type or other amino acids that did not affect fidelity (Rozen-Gagnon et al. 2014). These studies indicate that the polymerase fidelity of CHIKV is finely tuned to achieve a mutation frequency that optimizes fitness and adaptability in mosquitoes.

Prediction of CHIKV Evolutionary Trajectories

Analyses of CHIKV evolutionary history have underscored the importance of viral genetic factors for the emergence of novel CHIKV strains with enhanced epidemic potential. Most of the studies, however, have focused primarily on retrospective investigation of mutations associated with CHIK outbreaks. They are therefore limited in ability to predict current trends of the ongoing CHIKV epidemic, as well as to assess the probability of emergence of novel CHIKV strains capable of further global expansion. Moreover, as discussed above, a constellation of specific ecological factors is involved in determining the scope and duration of any given outbreak, making confident predictions of CHIKV evolutionary trajectories problematic.

Nevertheless, attempts have been made to elucidate future trends of CHIKV epidemics (Tsetsarkin et al. 2014; Stapleford et al. 2014). The sequential increase in CHIKV transmissibility by *A. albopictus* observed in IOL strains since 2005 supported the hypothesis that the evolution of this CHIKV trait will continue in the near future. Two approaches have been used to predict particular mutations with potential to emerge in CHIKV circulating in the human-*A. albopictus* cycle. Using deep sequencing analysis of CHIKV genomes present in saliva extracts from experimentally infected *A. albopictus* mosquitoes, the appearance of two mutations (E1-V80I and E1-A129V) was repeatedly observed (Fig. 5). Subsequent reverse-genetic experiments demonstrated that this double mutation E1-V80I/E1-A129V increases CHIKV infectivity and dissemination in *A. albopictus* and *A. aegypti* mosquitoes, and is also associated with increased CHIKV replication in C57BL/6 mice. Moreover this double mutant is selected during laboratory CHIKV transmission

between mice and *A. aegypti*, indicating that it has a potential of displacing parental CHIKV strains in the areas inhabited not only by *A. albopictus* but also by *A. aegypti*. However, a caveat of this study is that neither mutation has been observed in CHIKV isolated in nature (Stapleford et al. 2014).

A parallel study was built upon the observation that all natural second-step *A. albopictus*-adaptive mutations detected to date involve acquisition of glutamine in the ASR of the E2 protein. To determine if additional positions within ASR might be involved in CHIKV adaptation to this mosquito, 6 amino acids in the ASR were substituted with glutamine and/or glutamic acid, followed by fitness evaluation in *A. albopictus*. Substitutions in three positions, E2-233, E2-234, and E2-248, resulted in significant increases in CHIKV infection and dissemination (Fig. 5). These results suggest that emergence of novel *A. albopictus*-adapted CHIKV sublineages is likely in regions where *A. albopictus* is abundant (Tsetsarkin et al. 2014). Moreover, a combination of second-step *A. albopictus*-adaptive mutations E2-L210Q and E2-K252Q in a single genome resulted in a virus that was even more efficient in its ability to infect *A. albopictus* than any natural CHIKV strain studied to date. This suggests that further *A. albopictus*-adaptive evolution of CHIKV will favor the selection of "super-adapted" strains with even greater potential for global expansion as this vector continues to invade new regions (Tsetsarkin et al. 2014).

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Chikungunya Virus Entry and Replication

Nathalie Chazal and Laurence Briant

Introduction

The Chikungunya virus (CHIKV) is an enveloped virus organized with icosahedral symmetry of triangulation $T=4$, containing 80 spikes that make a glycoprotein shell enclosing the viral membrane and the nucleocapsid. The viral genome is a positive single-stranded RNA of 11.8 kb in size organized into two open reading frames. The 5′ end of the genome encodes a polyprotein that is ckeaved to give the four non-structural proteins (nsP1–nsP4) with enzymatic functions necessary for viral RNA replication and transcription. The structural proteins required for assembly and budding of a new viral particle, including capsid, E1 and E2 envelope glycoproteins, E3, and 6 K are encoded by a second ORF controlled by the internal subgenomic promoter. Despite being poorly addressed by direct studies, the replication cycle of CHIKV can be anticipated from the knowledge accumulated from the study of the Sindbis virus (SINV) and Semliki Forest virus (SFV) also members of the alphaviruses genus and *Togaviridae* family. *Alphavirus* replication takes place in the cytoplasm and is initiated by the synthesis of the nsP1234 precursor directly from the RNA genome (Fig. 1). Then, a progressive maturation process releases the free nsP4 RNA-dependent RNA polymerase that complexes with the uncleaved nsP123 polyprotein and host cofactors to catalyze the synthesis of the negative-strand RNA intermediate (Barton et al. 1991). As the replication processes, the fully cleaved nsPs act as a plus-strand RNA replicase to amplify the full-length positive-strand mRNA using the negative-strand RNA as a template (Shirako and Strauss 1994). Then, this RNA serves as a template for the production of the subgenomic RNA and the synthesis of the structural polyprotein that is processed cotranslationally and post-translationally into capsid and pE2-6K-E1 polyprotein. Maturation by furin

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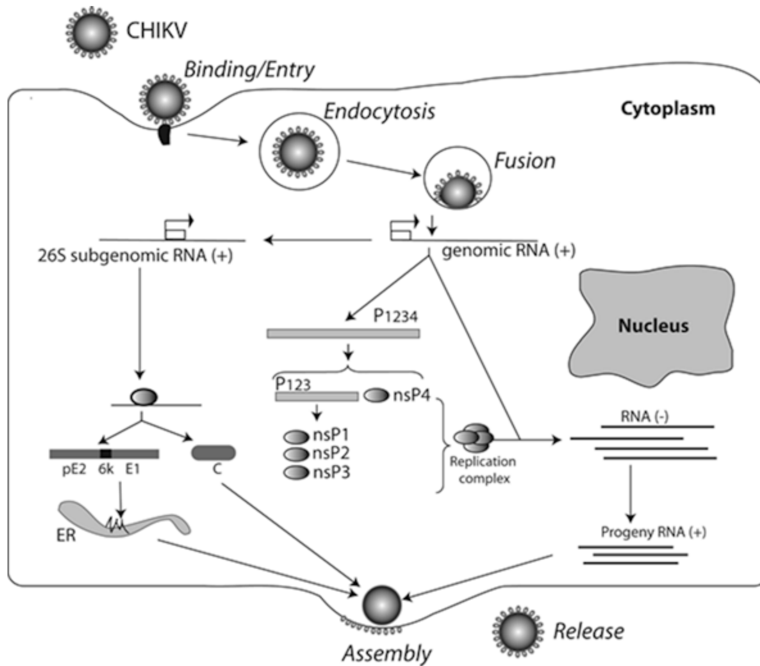

Fig. 1 Replication cycle of *alphaviruses*. Intracytoplasmic replication is initiated by expression of the nsP1234 precursor directly from the RNA genome. This precursor is maturated into: nsP1 which possesses both guanine-7-methyltransferase and guanylyl transferase activities required for capping and methylation of newly synthesized viral RNAs; nsP2, a cysteine protease required for processing of the nonstructural polyprotein that displays RNA triphosphatase/nucleoside triphosphatase and helicase activities; nsP3 a putative ADP-ribose 1-phosphate phosphatase; and nsP4, the RNA-dependent RNA-polymerase. All four proteins ensure genomic RNA replication through the synthesis of a negative-strand intermediate (RNA(−)) and drive expression of the structural polyprotein controlled by the internal subgenomic promoter. Capsid, a *cis*-acting autoprotease cleaves itself out of the structural polypeptide and releases the unprocessed pE2-6K-E1 envelope precursor that traffics to the plasma membrane through the secretory pathway. The *alphavirus* nucleocapsid is assembled by direct interaction of the oligomerized capsid protein with the viral RNA genome. The viral particle becomes enveloped by envelope glycoproteins inserted in the plasma membrane and recruited by the C-terminal moiety of the capsid protein. Finally, the nascent viral particle is released in the cytoplasm

and cellular convertases gives rise to E1, E2, E3 envelope glycoproteins and to a small peptide termed 6 K. The alphavirus particle assembly is supposed to start with capsid interaction with the RNA genome and requires capsid oligomerization. Budding of the viral particle in the extracellular medium is mediated by the final binding of the nucleocapsid to E2 (Suomalainen and Garoff 1992).

The completion of this replication cycle implies that the viral particle has initially attached to its cellular target. In this task, the high affinity binding of the viral envelope glycoproteins to specific receptor(s) can be assisted by a number of

attachment factors required for increasing and stabilizing virus–host interactions. The distribution pattern of these molecules on host cells in tissues defines the viral tropism in the infected host. Then, delivery of the viral particle to the appropriate compartment supporting viral entry will determine the success of the infection process. As viral entry represents the earliest drugable target in a virus life cycle, elucidating the host cell components required for attachment of medically important viruses to the cell surface and understanding how these pathogens enter their target cells represents a real challenge for the design of therapeutic strategies aimed at interrupting viral replication. The nature of cellular proteins and biological processes hijacked by CHIKV to penetrate into its target cells in vertebrates and mosquitoes is the scope of this review.

CHIKV Tropism in Vertebrate Host

The *alphaviruses* have a very wide host range and within their hosts they infect and replicate in a large variety of cells of different lineage leading to the infection of various organs. Since its reemergence in 2004 a number of studies questioned the capacity of CHIKV to replicate in immortalized and primary human cells originated from various tissues (Table 1). These studies established the capacity of CHIKV to grow in transformed hepatocytes (Solignat et al. 2009; Wikan et al. 2012), in fibroblasts and epithelial cells originating from lung (Wileman et al. 1984; Sourisseau et al. 2007), from kidneys (Sourisseau et al. 2007; Bernard et al. 2010), and from uterus (Sourisseau et al. 2007; Krejbich-Trotot et al. 2011; Solignat et al. 2009). Cells participating in bones and joints, namely osteoblasts (Noret et al. 2012) and primary fibroblast-like synoviocytes (Phuklia et al. 2013) together with diverse human muscular cells (immortalized skeletal myoblasts, primary muscle fibroblasts, RD rhabdomyosarcoma cells) (Kaur et al. 2013; Ozden et al. 2007; Sam et al. 2012) were identified as relevant targets for CHIKV in vitro. Cultures of fetal microglial cells, brain endothelial cells, and neurons were also found permissive for CHIKV replication (Solignat et al. 2009; Dhanwani et al. 2011; Sourisseau et al. 2007; Wikan et al. 2012; Abere et al. 2012; Wintachai et al. 2012). These results are in line with the detection of viral antigens in the corresponding organs of experimentally infected mice (Couderc et al. 2008; Schilte et al. 2010) and macaques (Labadie et al. 2010). They also corroborate observations performed using biopsies of CHIKV-infected patients (Ozden et al. 2007) and finally fit CHIKV-associated symptoms (Suhrbier et al. 2012). In addition to these observations, cells from the hematopoietic lineage including B lymphocytes and activated T lymphocytes are generally reported as poorly permissive to CHIKV (Solignat et al. 2009; Sourisseau et al. 2007) and primary monocytes and macrophages are positive for CHIKV antigens in infected patients experiencing very high viral loads (Her et al. 2010). In contrast with these results, a number of cell types were reported to be refractory to CHIKV infection. Indeed, primary dendritic cells (Sourisseau et al. 2007), differentiated myotubes (Ozden et al. 2007), and some immortalized cell lines including

Table 1 Cellular tropism displayed by CHIKV for human tissues and cell types

Tissue	Type	Cell	Viral replication	Envelope binding	References
Immune cells	Monocytes	THP-1, U937	–	+	Rathore et al. (2013), Salvador et al. (2009), Sourisseau et al. (2007), Wikan et al. (2012)
		Primary	+		Her et al. (2010), Teng et al. (2012)
	Dendritic cells	Primary	–		Sourisseau et al. (2007)
	Macrophages	Primary	+		Solignat et al. (2009), Sourisseau et al. (2007)
	T lymphocytes	H9	–	–	Solignat et al. (2009)
		Jurkat	+/–		Rathore et al. (2013), Sourisseau et al. (2007)
	Erythromyeloblastoid cells	K562	+/–		Rathore et al. (2013)
	B lymphocytes	B-420	+/–		Sourisseau et al. (2007)
Liver	Hepatocarcinoma cells	HepG2	+	+	Hu et al. (2014), Wikan et al. (2012)
		HUH7	++		Solignat et al. (2009)
Uterine	Cervical epithelial cells	HeLa	+++	+	Hu et al. (2014), Rathore et al. (2013), Salvador et al. (2009), Solignat et al. (2009), Sourisseau et al. (2007), Wikan et al. (2012)
Lung	Alveolar epithelial cells	A549	–	+	Salvador et al. (2009), Sourisseau et al. (2007)
	Bronchial epithelial cells	BEAS-2B	+++		Li et al. (2013), Sourisseau et al. (2007)
	Lung fibroblasts	MRC5	+		Rathore et al. (2013), Schilte et al. (2010), Sourisseau et al. (2007), Wikan et al. (2012)
Kidney	Epithelial cells	HEK 293T HEK 293	+++	+	Bernard et al. (2010), Her et al. (2010), Hu et al. (2014), Rathore et al. (2013), Salvador et al. (2009), Solignat et al. (2009), Sourisseau et al. (2007), Wikan et al. (2012)
	Fibroblasts	MEF	+		Schilte et al. (2010)

Bone/joint	Osteoblast	Primary	+		Noret et al. (2012)
	Synovial sarcoma cells	SW-982	–		Wikan et al. (2012)
	Fibroblast-like synoviocytes	Primary	+		Phuklia et al. (2013)
	Bone marrow enthodelial cells	ThBMEC	+		Sourisseau et al. (2007)
Muscle	Rhabdomyosarcoma cells	RD	+++	+	Sam et al. (2012), Lam et al. (2012), Salvador et al. (2009)
	Satellite cells	Primary	+	+	Ozden et al. (2007), Salvador et al. (2009)
	Skeletal myoblasts	HSMM	++		Kaur et al. (2013)
		LHCN-M2	+		Ozden et al. (2008)
	Myotubes	Primary	–		Ozden et al. (2007)
	Myofibers	Primary	+		Rohatgi et al. (2014), Ozden et al. (2007)
	Muscle fibroblasts	Primary	+		Rohatgi et al. (2014)
Brain	Glioblastoma cells	U-87 MG	+++		Abraham et al. (2013)
	Fetal microglial cells	CHME-5	+		Abere et al. (2012), Wikan et al. (2012), Wintachai et al. (2012)
	Neuroblastoma cells	SH-SY5Y	++		Dhanwani et al. (2012), Solignat et al. (2009)
	Endothelial cells	hCMEC/D3	–		Sourisseau et al. (2007)
Connective tissue	Fibroblasts	HS633T	++		Thon-Hon et al. (2012)
		HT1080	+	+	Thon-Hon et al. (2012), Salvador et al. (2009)
Skin	Skin fibroblast	Hs789Sk	++		Sourisseau et al. (2007)
		Primary	+		Schilte et al. (2010), White et al. (2011)
	Keratinocyte	HaCaT	–	+	Bernard et al. (2014)
		Primary	–	+	Bernard et al. (2014), Salvador et al. (2009)

hCMEC/D3 brain endothelial cells, SW982 synovial sarcoma cells (Sourisseau et al. 2007; Wikan et al. 2012), and the A549 alveolar epithelial cell line are unable to replicate CHIKV. The mechanisms accounting for CHIKV resistance remain uninvestigated in most cases. However, we recently reported that absence of replication in primary keratinocytes and in the HaCaT spontaneously immortalized keratinocyte cell line results from an intracellular block of the viral cycle because these cells support the replication of CHIKV envelope-pseudotyped retroviral particles and are unable to replicate wild-type CHIKV (Bernard et al. 2014). Again, these results are consistent with skin biopsies from experimentally infected animals showing that at the site of inoculation, viral antigens accumulate in the basal fibroblast skin layer instead of the keratinocytes-rich epidermal layer (Labadie et al. 2010; Schilte et al. 2010). In addition to this variety of human cells, epithelial cells originating from monkeys (Vero and LLC-MK2) (Higashi et al. 1967; Simizu et al. 1984), mice fibroblasts (BHK21 and L929) (Glasgow 1966; Davis et al. 1971), and chick embryos (White et al. 1972) were also described as permissive for CHIKV replication. These observations, together with the capacity of CHIKV to cycle alternatively between vertebrates and mosquitoes in which the virus replicates, attest for the wide cellular tropism of CHIKV and reflect its wide species range. They may also suggest the existence of cellular receptors highly conserved between species or instead may reflect the capacity of this pathogen to use multiple receptors in its various hosts.

Receptors and Attachment Factors

As animal viruses initiate infection of susceptible cells by binding to receptors expressed at the cell surface, the distribution of these molecules is a key determinant of the host range and tissue tropism. Early attempts to identify cellular proteins used receptors for *alphavirus* entry in human cells showed that virion binding to the cell surface is saturable and sensitive to protease digestion of the target cells, suggesting the requirement for membranous proteinaceous receptor(s) (Marsh and Helenius 1980; Smith and Tignor 1980). However, despite the considerable number of studies carried out during the last decades, the identity of host surface proteins hijacked for infection by *alphaviruses* and especially by CHIKV still remains unclear. The earliest studies implemented on SINV and SFV using a variety of strategies and especially anti-idiotypic antibodies as probes pointed out the contribution of various host molecules according to the cell target used. Proteins with a molecular weight of 74 and 110 kDa were proposed to mediate infection of mouse neural cells (Ubol and Griffin 1991) whereas candidate receptors of 63 and 90 kDa in size were reported to allow entry in avian cells (Wang et al. 1991) and human lymphoblastoid cells (Maassen and Terhorst 1981), respectively. Although the identity of these cell factors remains unknown, various proteins were proposed as candidate receptors for *alphaviruses* including the 67 kDa laminin reported as a high-affinity receptor for SINV and for the Venezuelan equine encephalitis virus (Wang et al. 1992; Malygin et al.

2009), the collagen-binding α1β1 integrin (CD49a/CD29) found to mediate infection of mammalian cells by the Ross River virus (La Linn et al. 2005) and the C-type lectins DC-SIGN and L-SIGN that conferred susceptibility of human monocytic and primary dendritic cells to SINV (Klimstra et al. 2003). The most recent data in this field proved that the natural resistance-associated macrophage protein (NRAMP), a proton-coupled divalent metal ion transporter across the cell membranes, identified from a RNAi screen mediates entry of laboratory adapted and pathogenic SINV strains in drosophila cells while its human homologue NRAMP2 mediates binding of SINV envelope glycoproteins and infection of murine and insect cells (Rose et al. 2011). In addition to these candidate receptors, the widely expressed negatively charged cell surface glycosaminoglycan heparan sulfate was proposed as an attachment factor enhancing the binding of viral particles to target cells (Byrnes and Griffin 1998; Bernard et al. 2000; Heil et al. 2001). A possible contribution in enhanced infection and increased virulence of circulating eastern equine encephalitis viruses (Gardner et al. 2011) and laboratory-adapted SINV strains bearing selective mutations for positively charged amino acid in E2 glycoprotein (Klimstra et al. 1998) was proposed for these host molecules (Zhu et al. 2010; Gardner et al. 2011). Regarding CHIKV, in the absence of specific antiviral therapy and given the potential therapeutic importance of strategies inhibiting receptor–envelope interactions, a specific effort has been performed to identify the putative receptor(s) and attachment factor(s) used by this virus. A combination of 2D virus overlay proteins binding assay (VOPBA) and mass spectrometric analysis identified the phosphoglycerate mutase 1 (PGAM), the 60 KDa heat shock protein (Hsp60), the far upstream element-binding protein 2 (FBP-2), and prohibitin 1 (PHB1) as CHIKV-binding proteins expressed at the surface of human microglial cells (Wintachai et al. 2012). Among these proteins, PHB1 was clearly shown to bind the E2 envelope glycoprotein but PHB1-specific siRNA and anti-PHB1 antibodies, despite reducing infection, failed to abolish viral entry (Wintachai et al. 2012). Accordingly, PHB1 may be at best one of the receptors used by CHIKV to infect microglial cells. In the very last years, Jemielity et al. (2013) proposed that the human T-cell immunoglobulin and mucin-domain 1 protein (hTIM1) mediating the entry of a broad range of viruses including filoviruses, flaviviruses, and New World arenaviruses may also act as a receptor for alphaviruses and especially for CHIKV. Indeed, overexpression of hTIM1 in human epithelial 293 T cells enhanced by 8-fold the entry of CHIKV-pseudotyped retroviral particles, an effect that could be reverted by preincubation of the cells with anti-hTIM1 mouse monoclonal antibodies. The hTIM1 ectodomain includes an amino-terminal variable immunoglobulin-like domain containing a high-affinity binding site for phosphatidylserine (PtdSer), a phospholipid found in the eukaryotic membranes and in alphavirus particles budded from their mammalian hosts. As CHIKV entry enhancement was not observed in cells overexpressing an hTIM1 variant unable to bind PtdSer, hTIM1 was proposed to promote infection by associating with PtdSer incorporated in the viral envelope (Jemielity et al. 2013). A similar effect could be recapitulated by ectopic expression of a glycosylphosphatidylinositol-anchored alpha-dystroglycan-annexin V chimera containing a PtdSer binding site in its annexin V N-terminal moiety (Moller-Tank and Maury 2014). However, incorporation of viral

envelope glycoproteins was found dispensable for enhancement of viral infection by PtdSer-binding proteins. Moreover, the efficiency with which CHIKV pseudoviruses utilize PtdSer receptors appears to be dependent on the cellular background because hTIM1 overexpression had lesser effects in 3T3 compared to 293 T cells and anti-hTIM1 antibodies only partially blocked infection of hTIM1-positive Huh7 human cell line (Jemielity et al. 2013). Altogether, these data indicate that several PtdSer-binding proteins behave as attachment factors rather than envelope-specific receptors and the nature of CHIKV-specific receptor(s) still remains to be elucidated.

Endocytosis-Dependent Internalization of CHIKV Particles

Once bound to cellular receptors expressed at the surface of susceptible cells, many different viral species depend on endocytic uptake of the receptor–virus complex for delivery to endosomes and penetration in the cytoplasm. Such internalization may occur through various mechanisms generally accounting for internalization, sorting, and absorption of extracellular macromolecules and membrane-associated receptors, including clathrin-mediated endocytosis, macropinocytosis, caveolar/lipid raft-mediated endocytosis, or by a variety of other still poorly characterized mechanisms (Yamauchi and Helenius 2013). Clathrin-mediated endocytosis is undoubtedly the best understood endocytic route hijacked by viruses. This endocytic pathway requires clathrin protein assembly as a coat for the nascent vesicle on the inner surface of the plasma membrane, allowing membrane invagination. Then, membrane fission and release of the coated vesicle from the plasma membrane is assisted by the large GTPase dynamin. Once internalized, the endocytic vesicle loses its clathrin coat and fuses with the endosome in which the viral particle is released. Maturation of the vesicle occurs during the microtubule-mediated movement of the vacuole towards the perinuclear region and converts the early endosomal vacuole decorated by the Rab5 a GTPase to the maturated Rab7-enriched late endosomes (for review see Yamauchi and Helenius (2013). This dynamic process is accompanied by a progressive acidification of the intralumen pH from 6.5 in early endosomes to 5 in late endosomes, governed by the H^+-ATPase generating a proton motive force across the plasma membrane. The acid-activated exposure of envelope fusion peptides due to low endosomal pH triggers the fusion of the viral particle with the limiting membrane of the endosome, resulting in nucleocapsid penetration in the cytoplasm and in the final release of the viral genome in an appropriate place supporting viral replication (Helenius et al. 1980; Mercer et al. 2010). According to the pH needed to activate membrane fusion, viruses preferentially use early or late endosomes for entry. The earliest evidence revealed that the prototypal alphavirus SFV hijacks the clathrin-dependent endocytic pathway to penetrate into vertebrate cells (Helenius et al. 1980; Marsh et al. 1984; White and Helenius 1980), a mechanism conserved for SINV entry in human epithelial cells (DeTulleo and Kirchhausen 1998). However, some controversy arose from the evidence that in some experimental conditions, inhibition of endosomal acidification blocks SINV RNA

synthesis without inhibiting virus entry into target cells (Cassell et al. 1984; Hunt et al. 2011). Together with the capacity of SINV to infect hamster CHO cells defective in endosomal acidification (Edwards and Brown 1991), these results prompted the hypothesis that at least some *alphaviruses* may infect their host via an endocytosis-independent mechanism. In a recent study Kononchik et al. (2011a) argued that *alphaviruses* especially SINV, may take advantage of pores created at the plasma membrane probably through the 6 K protein, to inject their nucleocapsid into the host. Electron micrographs showing empty particles connected at the cell surface reinforce this model (Paredes et al. 2004; Kononchik et al. 2011b; Vancini et al. 2013). During this process, conformational glycoprotein changes required for viral fusion would be directed by receptor binding instead of the acidic environment. Despite such evidence provided for SINV, the possibility of applying this model to CHIKV yet remains unresolved. Instead, electron microscopy analysis together with immunofluorescence studies revealed that entry through endocytosis evidenced for *alphaviruses* is conserved for CHIKV (Solignat et al. 2009; Bernard et al. 2010; Fig. 2). However, despite infection being inhibited by a mutant form of the AP2-associated adaptor Eps15 protein that functions as a clathrin accessory protein but also contributes to the coupling of ubiquitinated cargo to clathrin-independent internalization (Sigismund et al. 2005), abolition of clathrin heavy chain expression by RNAi surprisingly had no effect on CHIKV infection of 293T cells (Bernard et al. 2010). Accordingly, although SINV and SFV entry was reported to be strictly dependent on clathrin expression (DeTulleo and Kirchhausen 1998; Ooi et al. 2013), CHIKV penetration in the host cell could use alternative routes in clathrin-deficient epithelial cells. As observed for SFV and SINV (DeTulleo and Kirchhausen 1998) inhibition of the cellular GTPase dynamin that regulates the scission step of endocytic vacuoles from the plasma membrane dramatically reduced CHIKV internalization and subsequent replication (Bernard et al. 2010; Sourisseau et al. 2007). Also, chemical agents disrupting the microtubule network or inducing the depolymerization of actin fibers that drive the movement of the endocytic vacuole to the deeper cytoplasm had a profound inhibitory effect on CHIKV replication that could not be bypassed by acid-mediated activation of viral particles attesting that integrity of actin fibers and microtubules is not only required for viral internalization, but also for a post fusion step of the viral life cycle (Bernard et al. 2010). The acidic pH in endosomal vacuoles is the next requirement for exposure of CHIKV fusion peptides before virus–cell membrane fusion and cytosolic delivery of the RNA genome into the cytoplasm. Inhibition of endosomal vesicles acidification by bafilomycin A1, which is a specific inhibitor of vacuolar type H^+-ATPase, as well as NH_4Cl and chloroquine that also interferes with endosome acidification equally inhibited CHIKV infection (Bernard et al. 2010). This result is consistent with the pH activation experiments indicating that SFV fusion requires an acidic environment and with the inhibition of SFV entry and infection by endosome acidification inhibitors (nigericin, concanavalin A) (Irurzun et al. 1997; Helenius et al. 1980, 1982; Marsh et al. 1983). However, pH requirements vary according to the virus considered: optimal pH for SFV fusion is 6.2 (White and Helenius 1980; Glomb-Reinmund and Kielian 1998) whereas the fusion threshold varies markedly

from pH 5.6 to 6.5 according to the SINV strain considered (Glomb-Reinmund and Kielian 1998; Smit et al. 1999). This pH activation threshold argues for a SFV fusion step occurring in early endosomes, an hypothesis that was further confirmed in cells expressing the dominant negative (DN) Rab5 GTPase (Sieczkarski and Whittaker 2003; Vonderheit and Helenius 2005). This property is likely conserved for CHIKV inasmuch as infection was unable to proceed in human epithelial cells expressing DN Rab5 proteins (Bernard et al. 2010) although unaffected by expression of a DN Rab7 mutant. In the endosomal compartment, the low pH induces local conformational changes at the virus surface characterized by E1 release from the E1/E2 heterodimer (Helenius et al. 1980; Justman et al. 1993; Bron et al. 1993; Klimjack et al. 1994; Smit et al. 1999; White and Helenius 1980). E1 at the monomeric state then inserts its hydrophobic fusion peptide in the host cell membranes before formation of homotrimers active for fusion (Cao and Zhang 2013; Liu and Kielian 2009; Corver et al. 1997). Membrane lipids, especially sphingolipids, more precisely galactosylceramide, and cholesterol are required for fusion directed by alphavirus E1 glycoprotein (Phalen and Kielian 1991; Nieva et al. 1994; Smit et al. 1999; White and Helenius 1980; Wilschut et al. 1995; Ahn et al. 2002; Lu et al. 1999). However, lipid rafts frequently used as platforms for virus entry do not contribute to SFV or SINV infection (Waarts et al. 2002). The essential function fulfilled by membrane lipids is supposed to be conserved for CHIKV as viral propagation in cultures is disrupted by the cholesterol depletion agent methyl-β cyclodextrin (Bernard et al. 2010). In addition to these well-known pathways, the precise role played by several other candidate proteins identified in a recent genome-wide RNAi screen as cofactors for *alphaviruses* entry remain to be validated in the context of CHIKV infection. This is especially the case for the fuzzy homologue FUZ and tetraspanin 9 both required for SINV infection and transferrin endocytosis (Ooi et al. 2013; Collinet et al. 2010). Accordingly molecular mechanisms of CHIKV entry still deserve attention.

Mechanisms of CHIKV Entry in Mosquito Cells

As *alphaviruses* efficiently replicate in *Aedes* mosquitoes, a focus was also given to entry mechanisms in insect cells. The first events occurring in mosquitoes after ingestion of a blood meal from a CHIKV-infected vertebrate consist of replication of the ingested virus in the midgut cells followed by colonization of the salivary glands (Ziegler et al. 2011). Due to the absence of informative immortalized cell lines, viral tropism for mosquito tissue remains poorly explored. Instead, CHIKV was found to replicate in the immortalized C6/36 cell line derived from larvae of the *Aedes albopictus* competent mosquito vector (Singh 1967) and in Aa23 or CCL-125 *Aedes aegypti* cells (Vavre and Mavingui 2011; Wikan et al. 2012). Several CHIKV putative receptors were identified in the brush border membrane fraction of *Aedes aegypti* mosquitoes (proteins of 60 and 38 kDa in size) (Mourya et al. 1998) and in the membrane fraction of C6/36 cells (proteins of 24, 45, 58, and 62 kDa; Mourya

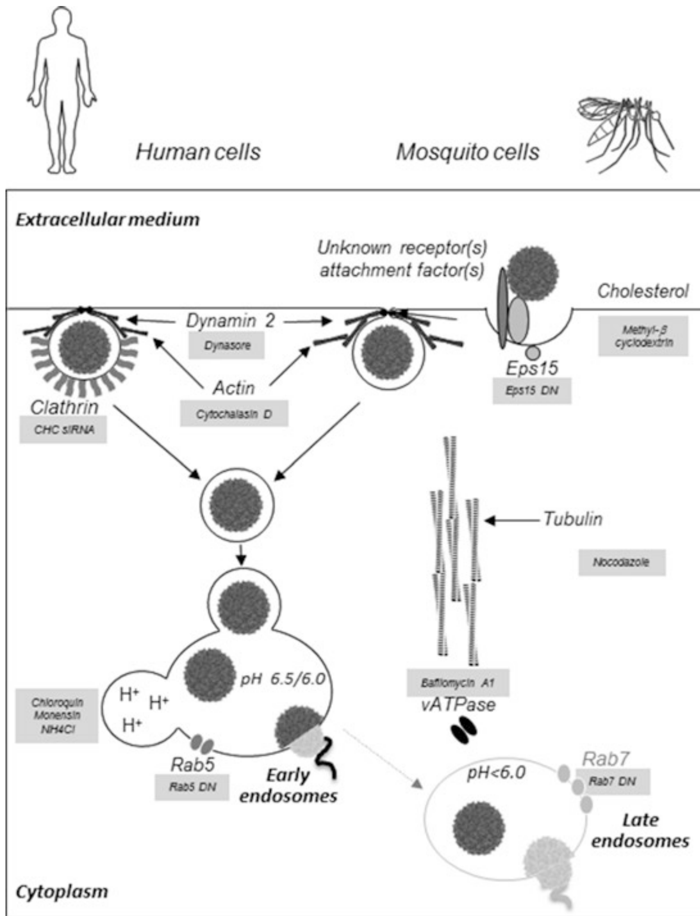

Fig. 2 Endocytic pathways used by Chikungunya virus to infect human and mosquito cells. Cellular proteins assisting endocytosis of CHIKV are shown in italic and inhibitors used to decrypt these pathways are highlighted in grey

et al. 1998). More recently, VOPBA strategies and mass spectroscopy identified the 50 kDa ATP synthetase β subunit (ATPSβ) as a CHIKV-binding protein (Fongsaran et al. 2014). Coimmunoprecipitation and colocalization experiments confirmed this interaction at the cell surface and a significant reduction in CHIKV entry was reported with both antibodies and siRNA targeting ATPSβ suggesting that ATPSβ mediates CHIKV entry in C6/36 and CCL-125 cells.

As for vertebrate cells, several recent studies investigated the endocytic routes recruited by CHIKV to infect mosquito cells (Fig. 2). These studies performed using the C6/36 cells line proved that the picture of entry events drawn from vertebrate cells is mostly conserved in insect cells. Especially, dynamin, Eps-15, and actin as well as association of viruses with the Rab5-positive early endosomal com-

partment are required for viral entry in these cells (Lee et al. 2013). Acidification of the endosomal pH is also mandatory as well as expression of the V-ATPase (Gay et al. 2012; Lee et al. 2013; Nuckols et al. 2014). Finally, cholesterol depletion either by means of addition of Cab-O-Sil-delipidated serum to the cell culture or by methyl-β cyclodextrin treatment reduces infection (Lee et al. 2013; Gay et al. 2012). Sensitivity to these agents is, however, modulated by genetic variations in E1 envelope glycoprotein as discussed below. Of note a systematic transcriptomic microarray analysis revealed that epsin1 and huntingtin-interacting protein participating in clathrin-mediated endocytosis are upregulated in CHIKV-infected C6/36 cells (Lee et al. 2013) suggesting their possible contribution in CHIKV entry in a way that remains to be determined.

Viral Entry and Fitness

The comparison of CHIKV variants isolated from various epidemic waves revealed different sensitivity to drugs perturbing viral entry. In 2004, the emergence of CHIKV in Kenya and subsequent spread in Indian Ocean Islands, was associated with the acquisition of a A-to-V 226 mutation in the E1 envelope glycoprotein of an East-Central-South African strain (Schuffenecker et al. 2006). This mutation was sufficient to confer a preferential selection by *Aedes albopictus* midgut barrier, allowing an increased replication, a more efficient dissemination to secondary organs (wings and salivary glands), and an improved transmission to mice. Conversely, the native E1-226A variant was slightly better disseminated in *Aedes aegypti* and more efficiently transmitted by this vector (Tsetsarkin et al. 2007; Arias-Goeta et al. 2013; Dubrulle et al. 2009). In vitro, the E1-226V mutant replicated more efficiently in C6/36 and CCL-125 *Aedes albopictus* cells therefore corroborating mosquito infection experiments. The E1-226V variant displayed a greater membrane cholesterol dependence (Gay et al. 2012; Tsetsarkin et al. 2007; Wikan et al. 2012) that was correlated with the localization of the mutated residue in the E1 fusion loop required for cholesterol binding conserved among alphaviruses (Schuffenecker et al. 2006; Voss et al. 2010; Umashankar et al. 2008). Despite an association between cholesterol dependence and increased viral fitness in *Aedes albopictus* was first proposed (Tsetsarkin et al. 2007), a systematic mutational analysis contradicted this result (Tsetsarkin et al. 2011). Interestingly, E1-226V CHIKV variants were also found slightly more sensitive to endosomal pH inhibitors (Gay et al. 2012; Lee et al. 2013). This mutation was further confirmed to account for a decrease in pH threshold required for fusion events, with the E1-226V mutation conferring a ~0.2 lower pH sensitivity than observed with the E1-226A virus (Tsetsarkin et al. 2011). Both the lipid composition and the intravacuolar pH are known to vary according to the endosome maturation state. Accordingly, because recently propagated E1-226V CHIKV strains displayed some particular features regarding entry with a higher dependence upon membrane cholesterol and an increased requirement for low endosomal pH, the impact of genetic variations in E1

on the vesicular compartment used for fusion may be considered. In vertebrate cells, no significant difference in replication or cytopathogenicity was generally associated with the amino acid at position 226 (Wikan et al. 2012). The E1-226V mutant was, however, reported to replicate more efficiently in neuroblastoma cells and to generate lower expression of IFN-β; Toll-like receptors 3 and 7; and MX-2, ISG-15, and 2′,5′-oligoadenylate synthetase 3 (OAS-3) antiviral genes as compared with E1-226A CHIKV (Priya et al. 2013). Together with the higher induction of IL-10, an anti-inflammatory and immunosuppressive cytokine induced by this variant in neuroblastoma cells, these data may argue for an increased capacity of the E1-226V CHIKV to escape host defenses, a mechanism proposed to participate in the increased pathogenesis of the re-emerged CHIKV (Priya et al. 2013). Additional information supporting cross-talk between viral entry and innate immune responses were produced by the study of a E2-E166K CHIKV variant (Henrik Gad et al. 2012). This mutation in the acid-sensitive region in E2 glycoprotein enhances CHIKV infectivity and cytopathogenicity in human epithelial cells and myoblasts. E2-E166K mutation also confers resistance to OAS3, an interferon inducible protein that targets the viral RNA to RNase L for degradation, and subverts the PKR-dependent antiviral pathway. Altogether, these studies point out a possible correlation between genomic mutations in envelope glycoproteins, the dynamic of the earliest events of the viral life cycle, and the cellular compartment where these events occur together with the capacity of the virus to stimulate or to subvert the host antiviral responses.

Viral Entry and Therapeutic Issues

As reported above, the entry of virus in the host cell involves several successive steps, each representing a potential target for therapeutic intervention. The first attempts to block *alphavirus* entry relied on the possibility to inhibit acidic-mediated fusion using chloroquine. This antimalaria drug was first reported to inhibit SINV and SFV infectivity in vitro more than 40 years ago (Helenius et al. 1982; Coombs et al. 1981; Cassell et al. 1984; Inglot 1969; Shimizu et al. 1972). In recent years, several studies confirmed the capacity of chloroquine to inhibit CHIKV replication and associated cytopathic effect in vitro (Sourisseau et al. 2007; Bernard et al. 2010; Brighton 1984). A dose-dependent inhibition of CHIKV replication by chloroquine showed an EC_{50} values of 7 μM (Khan et al. 2010), a concentration very similar to the plasma levels detected during treatment of acute malaria. These studies pointed to chloroquine as a potentially promising anti-CHIKV molecule, however, these results were counterbalanced by its rather narrow therapeutic index because chloroquine fully inhibited CHIKV infection at 10 μM but was toxic at 100 μM (Sourisseau et al. 2007). Moreover, a double-blind placebo-controlled randomized trial conducted in CHIKV-infected volunteers at the end of the 2006 outbreak in La Réunion Island showed no significant clinical or biological difference between chloroquine-treated patients and those receiving placebo in terms of symptoms duration or viremia (De Lamballerie et al. 2008). Instead, the long-term

follow-up of these volunteers showed that at day 200 of treatment, chloroquine-treated patients complained more frequently of arthralgia than those receiving placebo. In a second trial, chloroquine treatment provided no benefit compared with the nonsteroidian anti-inflammatory drug meloxicam in the management of musculoskeletal pain and regarding circulating levels of selected cytokine (IL-6, IFNγ, TNFα, CXCL10/IFNγ-inducible protein 10, and IL-13) during acute infection (Chopra et al. 2014). This lack of efficiency was finally confirmed by Padmakumar et al. (2009) who reported that coadministration of hydroxychloroquine with nonsteroidian anti-inflammatory drugs in acute stages of the disease does not offer any additional benefits. Altogether, these studies do not support a meaningful therapeutic role of chloroquine in the management of acute infection and there is still an urgent need for the discovery of anti-CHIKV molecules. Among candidates reported so far, arbidol (1-methyl-2-phenyl-thiomethyl-3-carbotoxy-4-dimethylaminomethyl-5-hydroxy-6-bromoindolehydrochloride) blocks the earliest stages of the replication cycle in Vero and MRC-5 cells ($IC_{50} < 10$ μg/mL) (Delogu et al. 2011). Originally developed at the Russian Research Chemical and Pharmaceutical Institute about 20 years ago and used since 1990 for prophylaxis and treatment of acute respiratory infections including influenza, arbidol exhibits a wide range of activity against a number of RNA and DNA enveloped and nonenveloped viruses (Boriskin et al. 2006) suggesting its capacity to target common critical step(s) in virus–cell interaction. Recent data showed that arbidol incorporates into cellular membranes leading to perturbed membrane structures and inhibition of virus-mediated fusion (Villalain 2010). Arbidol selection pressure generated a single amino acid G407R substitution in the A domain of the E2 glycoprotein postulated to be involved in binding to host receptor(s) (Voss et al. 2010) suggesting that its antiviral activity may relate to inhibition of virus adsorption on target cells. Interestingly, arbidol was also recently reported to inhibit dynamin-2-induced membrane scission (Blaising et al. 2013) and may therefore inhibit CHIKV entry in many ways. This promising anti-CHIKV drug lead was subjected to several optimization rounds that resulted in the production of indole-based derivatives with increased selectivity index and lower cell toxicity (Di Mola et al. 2014). Several other molecules preventing endocytosis at the plasma membrane were also reported as CHIKV inhibitors. This was particularly illustrated by molecules sharing a common 10*H*-phenothiazine core structure including chlorpromazine, ethopropazine, methdilazine, perphenazine, thiethylphenazine, and thioridazine that are both effective anti-CHIKV molecules and clathrin-mediated endocytosis inhibitors (Pohjala et al. 2011). This was also recently confirmed for epigallocatechin-3-gallate (EGCG), the major constituent and most important catechin in green tea that possesses an antiviral activity against a broad range of viruses including CHIKV (Weber et al. 2015; Steinmann et al. 2013) and inhibits transferrin endocytosis (Huang et al. 2014). Alternatively, this molecule may also inhibit CHIKV attachment as recently proposed (Weber et al. 2015). Finally, as for many other viruses, the development of neutralizing monoclonal antibodies (mAbs) was considered as a very promising anti-CHIKV strategy. The alphavirus E2 envelope glycoprotein is involved in binding to host cell receptors and contains critical binding sites for

neutralizing antibodies with antiviral activity (Mendoza et al. 1988; Stanley et al. 1986). A recent study described the generation and characterization of two conformation-dependent mAbs (1.3A2 and 4.6F5) specific for CHIKV E2 (Goh et al. 2013). Concomitantly, the C9 mAb that recognizes a conformation-specific epitope mapping to the acid-sensitive region of E2 was produced from an antibody isolated from a CHIKV-infected patient (Selvarajah et al. 2013). These three mAbs were able to neutralize CHIKV in vitro and in vivo and provided protection against arthritis when used prophylactically in a CHIKV mouse model (Goh et al. 2013; Selvarajah et al. 2013). Although the use of neutralizing antibodies is costly and therefore inapplicable to treat significant portions of a population, these molecules could be useful to prevent infection of highly susceptible individuals (pregnant women, infants, and older individuals) during a CHIKV epidemic, to treat CHIKV-exposed individuals, and in combination with nonspecific antiviral molecules to attenuate disease severity in patients suffering from long-lasting, CHIKV-associated arthritis. These molecules therefore confirm the effectiveness of strategies targeting viral entry in the development of anti-CHIKV preventive or curative strategies.

In conclusion, the current knowledge reviewed in this chapter indicates that CHIKV entry in host cells is assisted by a complex virus–host crosstalk. The efficacy of this interplay has a significant impact on subsequent steps of the virus life cycle, on virus propagation into its host, and finally on viral pathogenesis. Although several key steps of CHIKV entry have been widely explored, several questions are still pending. The nature of cell receptors used by this virus to infect mammalian and mosquito cells still remains to be elucidated. The existence of clathrin-independent pathways and their exact contribution in infection is unclear. Finally, cross-talk between receptor binding/entry routes and innate immune responses pointed out in several recent studies remains to be defined. Elucidation of these aspects through the combination of cellular and biochemical studies with structural information, especially regarding envelope glycoproteins will be an important issue in the development of targeted and specific inhibitors of CHIKV entry and infection.

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Chikungunya Virus-Induced Autophagy and Apoptosis

Delphine Judith, Thérèse Couderc, and Marc Lecuit

This chapter focuses on two major host responses recently found to be involved in CHIKV infection: autophagy and apoptosis. For each process, we first present molecular pathways and associated signalling, then we highlight the diverse strategies developed by host cells to prevent viral replication and virus-induced cell death, as well as by the virus to fight and hijack these host cell defence pathways.

Autophagy Pathways and CHIKV

Autophagy Pathway

Autophagy is an intracellular degradative process highly conserved among eukaryotic cells that allows cells to recycle existing organelles and cytosolic components (Kuma and Mizushima 2010). It is required for cell development and survival of

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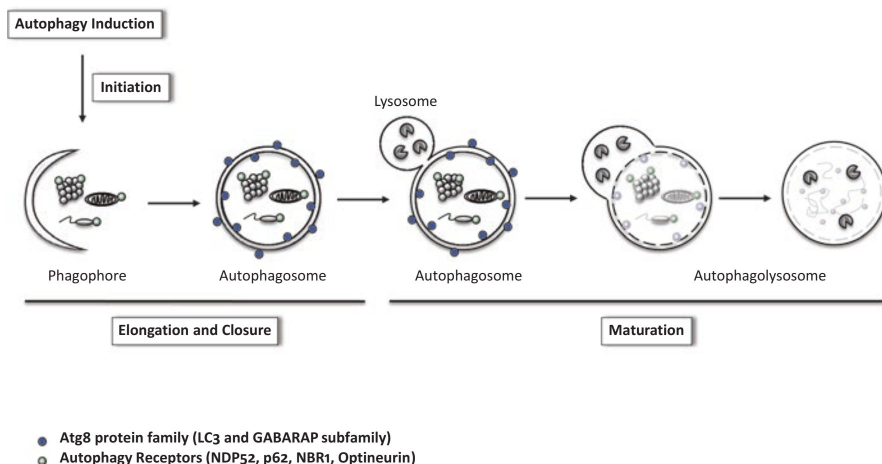

Fig. 1 Schematic representation of the nonselective and selective autophagy process. Autophagy is an evolutionarily conserved catabolic process in which intracellular material can be sequestered within double-membrane vesicles and targeted for degradation to lysosomes. Although autophagosomes can sequester cytosolic material nonspecifically in response to starvation (**a**), there is increasing evidence for selective autophagic degradation of various cellular structures, including protein aggregates, mitochondria, and pathogens (**b**). The selective autophagy process implicates autophagy receptors that mediate the docking of cargo to autophagosomes

eukaryotes and has an impact on cell homeostasis, tumorigenesis, neurodegeneration, cancer, diabetes, and infection (Choi et al. 2013). It represents the primordial form of eukaryotic innate immunity against invading microorganisms (Deretic et al. 2013).

The autophagic process is initiated by the formation of a double-membrane vesicle surrounding cytosolic materials to be degraded, including proteins and organelles, to form an autophagosome. Then, fusion of the autophagosome with the endo-lysosomal compartment leads to an autophagolysosome. This process consists of three different steps, which require autophagy-related genes (Atgs) and organelles and involves complex interactions between dedicated protein machinery and subcellular organelles (Lamb et al. 2013; Fig. 1). The molecular machinery includes more than 30 Atgs, discovered in yeast, at least 18 of which are required for mammalian autophagy (Mizushima et al. 2011). The first step, called initiation, corresponds to the formation of the autophagic isolation membrane or phagophore (Mizushima 2010; Chan 2009). The second step includes the elongation and expansion of the phagophore that occur from multiple membrane sources (Lamb et al. 2013; Hamasaki et al. 2013) through an unknown process but likely by vesicular delivery, followed by the closure and completion of a double-membrane autophagosome. The elongation and closure are controlled by members of the Atg8 ubiquitin-like protein family (Geng and Klionsky 2008). The Atg8 ubiquitin-like protein family includes LC3 (LC3A, LC3B (referred to as LC3 henceforth), LC3C) and GABARAP subfamilies (GABARAP, GABARAPL1, and GABARAPL2). The

soluble form of LC3 (referred to as LC3 hereafter) is termed LC3-I and the conjugated form LC3-PE as LC3-II. The LC3 conversion is widely used as a marker of autophagy flux (Klionsky et al. 2012). The last step is the maturation where the newly formed autophagosome fuses with endosomal compartment and/or with lysosomes to form the autophagolysosome.

Autophagy was previously described as a nonselective process but cumulative evidence has demonstrated its selectivity in recycling organelles, removing protein aggregates, and clearing specific viral proteins. Upon selective autophagy, autophagy receptors and the ubiquitination of the target are critical (Kirkin et al. 2009). Autophagy receptors are adaptor proteins, generally containing an ubiquitin-binding association domain (UBA) and an LC3-interaction region (LIR). Autophagy receptors can mediate the docking of ubiquitinated cargo to autophagosomes, thereby ensuring their selective degradation. The main autophagic receptors include p62 (SQSTM1), NBR1 (neighbour of BRCA1 gene 1), NDP52 (nuclear dot protein 52 kDa), and optineurin (Behrends and Fulda 2012). p62 is the best-characterized autophagy receptor and has been shown to target bacteria as well as viruses (Orvedahl et al. 2010; Mostowy and Cossart 2012).

Since the early reports, further studies have investigated the interplay between autophagy and viral infection and described that the autophagic process can be a host defence mechanism that clears intracytoplasmic viral products. However, viruses are able to subvert the autophagy machinery to favour their replication and release (Chiramel et al. 2013). Components of the autophagy machinery can therefore exert both an anti- or a pro-viral role, depending on the virus and the cell type considered (Dong and Levine 2013).

CHIKV Activates Autophagy

The evidence for the implication of the autophagy machinery during CHIKV infection, in cell cultures and in vivo, has been reported by several groups (Krejbich-Trotot et al. 2011; Judith et al. 2013; Joubert et al. 2012).

CHIKV infection induces autophagy as measured by the increased number of autophagosomes in infected human kidney epithelial cells (Krejbich-Trotot et al. 2011). Subsequent studies conducted by Judith et al. and by Joubert et al. showed that CHIKV infection triggers the conversion from LC3-I to LC3-II, a hallmark of the autophagy process, in primary and immortalised human cells as well as in mouse cells (Judith et al. 2013; Joubert et al. 2012). Analysis of the autophagy flux in the presence of lysosomal inhibitor and identification of autophagosomes and autolysosomes have proven evidence that CHIKV infection induces de novo autophagosome formation and that autophagosomes can fuse with lysosomes in CHIKV infected cells (Joubert et al 2012). Moreover, CHIKV infection decreases the level of p62, an autophagy receptor used as a marker for autophagic flux, providing evidence that CHIKV activates a complete autophagic response ending by the lysosomal degradation of the autophagic vesicle contents (Judith et al. 2013).

Although some viruses induce viral replication-independent autophagy, in most cases, autophagy induction by viruses is replication dependent, and initiated by a signal triggered either by viral replication steps, including entry and replication, or by accumulation of viral components or replication intermediates during the viral cycle. Indeed, this is the active CHIKV replication that induces autophagy, as it is not induced in cells treated with UV-inactivated CHIKV (Joubert et al. 2012). CHIKV promotes autophagy both by induction of endoplasmic reticulum (ER) stress and increase of reactive oxygen species (ROS) production (Joubert et al. 2012). ER stress is increased during viral infection and activates the unfolded protein response (UPR), which in turn induces autophagy. The UPR involves three different signalling pathways controlled by three integral ER membrane proteins: PERK, IRE-1α, and ATF6 (Hetz 2012). During CHIKV infection, accumulation of viral proteins in the ER may be the cause of ER stress, via an IRE1α- and XBP1s-mediated signalling pathway. ROS accumulation is a well-characterised host response to viral infections and free ROS are known to induce autophagy (Filomeni et al. 2014). CHIKV- induced ROS production induces autophagy through the inhibition of mTORC1. Both stress pathways act in an interdependent manner to enhance autophagic flux in CHIKV-infected cells (Joubert et al. 2012).

Antiviral Effect of Autophagy on CHIKV Infection

Xenophagy is a type of autophagy characterised by degradation of intracellular pathogens, helping to reduce their replication and spread. This type of autophagy involves selective recognition of pathogens that is ensured by particular autophagy receptors, such as p62 and NDP52 (Mostowy and Cossart 2012).

Judith et al. established direct antiviral roles for autophagy against CHIKV both in human and mouse cells. They found that CHIKV engages the molecular machinery of autophagy in a selective manner to protect infected cells (Fig. 2). By studying the implication of p62 in CHIKV infection, they found that the depletion of p62 significantly increased viral replication providing evidence that p62-mediated autophagy limits viral replication. They demonstrated that CHIKV capsid exhibits a cytotoxic effect and that the clearing of CHIKV capsid by p62 likely decreases its cellular toxicity, thereby limiting virus-induced cell death. They showed that by binding to LC3B, p62 recruit CHIKV capsid to the autophagosome in an ubiquitin-dependent manner and a SMURF1-independent manner, which degrade CHIKV capsid upon their fusion with lysosomes. Similarly, an earlier study was able to demonstrate the involvement of xenophagy during Sindbis virus (SINV) infection (Orvedahl et al. 2010). It has been reported that p62 delivered SINV capsids to degradation in autophagosome. However, even if SINV belongs to the same alphavirus genus as CHIKV, the signal recognition for the targeting of its capsids remains uncertain because, as opposed to CHIKV, it was reported to occur in an ubiquitin-independent manner but SMURF1-dependent mechanism. These observations raise questions regarding the status of CHIKV capsid (i.e., protein monomers or aggregates or assembled capsids), which is selectively targeted for autophagic degradation.

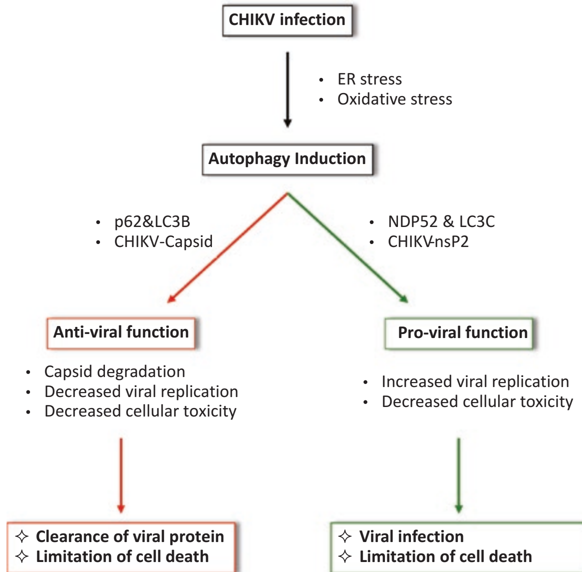

Fig. 2 Antiviral and pro-viral effects of the autophagy machinery upon CHIKV infection. Viral replication upon CHIKV infection induces both oxidative and endoplasmic reticulum stress leading to the induction of the autophagy process. The autophagy process can play either an anti- or a pro-viral role upon CHIKV infection. The antiviral role of the autophagy process involves the autophagy receptor p62 and the autophagic protein LC3B. By targeting to degradation the toxic CHIKV-capsid, p62 facilitates its clearance by the autophagy process leading to the limitation of cell death. The pro-viral role of the autophagy process involves the autophagic receptor NDP52 and the autophagic protein LC3C. By binding to LC3C and the CHIKV-nsP2, NDP52 promotes viral infection and limits cell death

Pro-Viral Effect of the Autophagy Machinery on CHIKV Infection

Krejbich-Trotot et al. investigated the effect of CHIKV-induced autophagy on viral replication and found that overall it promotes CHIKV viral replication in human kidney epithelial cells. They showed that impairment of the autophagy machinery reduces CHIKV replication whereas its induction enhances it (Krejbich-Trotot et al. 2011). The same phenotype is observed in HeLa cells, where depletion of canonical mediators of autophagy, Beclin1 and Atg7, decreases CHIKV replication (Judith et al. 2013). During CHIKV infection, nonstructural CHIKV proteins (nsPs) bind to viral RNA to form replicative complexes (RC). Among them, CHIKV nsP2 has been shown by high-throughput yeast two-hybrid (HT-Y2H) assay (Bourai et al. 2012) to interact with NDP52, and the depletion of NDP52, similarly to that of canonical mediators of autophagy, decreases CHIKV replication (Judith et al. 2013; Fig. 2). This suggests that CHIKV nsP2 may engage the autophagy machinery to help virus replication through the binding of NDP52, in human cells.

Further studies have shown that NDP52 associated with both LC3C and CHIKV nsP2, localizes to the *trans*-Golgi network-associated RCs that contain the other nsPs and double-stranded (ds)RNA replicative intermediate, in the vicinity of de novo protein synthesis (Judith et al. 2013). These observations suggest that NDP52 binding to CHIKV nsP2 and LC3C allows the anchorage of RCs to the TGN membrane.

However, one important result to consider is that mouse NDP52, in contrast to its human orthologue, is unable to bind to CHIKV nsP2, and LC3C is not expressed in mouse cells, accounting for the absence of promoting effect of the autophagy machinery on CHIKV infection in mouse cultured cells. The pro-viral role mediated by NDP52 is revealed by introducing human NDP52 and human LC3C in mouse cells, providing evidence of the species specificity of the pro-viral role of autophagy on CHIKV infection (Judith et al. 2013).

Apoptosis Pathway and CHIKV

Apoptosis Pathway

Apoptosis is highly conserved through evolution and is involved in the regulation of embryogenesis, development, and homeostasis by eliminating superfluous cells along these processes. Apoptosis can also be activated by a large number of stimuli as cell cycle perturbation, lack of nutrients, and viral infection. It is characterised by specific morphological features notably condensation and fragmentation of the nucleus, fragmentation of the mitochondrial network, and appearance of membrane blebs and apoptotic bodies (Taylor et al. 2008; Kerr et al. 1972).

The apoptosis process relies on the activation of cysteine aspartyl proteases known as caspases. Caspases are a conserved family of enzyme essential for initiation and execution of the apoptosis process. Caspases are central players in apoptosis because they catalyse many steps in the death pathway by irreversible cleavage of their substrates after aspartic acid residues. They are present as catalytically inactive proenzymes that are coordinately activated by caspase-specific cleavage. Two general classes of apoptotic caspases exist: initiator caspases including caspases 2, 8, 9, and 10, and effector caspases, which include caspases 3, 6, and 7. The initiator caspases are autoactivated under apoptotic condition, whereas effector caspases are activated in cascade through cleavage by initiator caspases. Effector caspases cleave a number of specific substrates, including structural components and regulatory proteins, leading to the destruction of cell–cell interactions and of the nuclear structure, reorganisation of the cytoskeleton, and inhibition of DNA synthesis (Kurokawa and Kornbluth 2009).

Apoptosis can be activated either by extrinsic or intrinsic stimuli. The extrinsic pathway is mediated by death receptors such as TNF receptors. Binding of the ligand to its death receptors induces a conformational change in the intracellular receptor domain that leads to the recruitment of apoptotic proteins to form the

DISC (death inducing signalling complex, downstream of FASL/TRAIL) or complex I (downstream of TNFR). The inactive initiator caspase-8 is recruited to the DISC and subsequently activated, leading to the initiation of the apoptosis process (Wilson et al. 2009).

The intrinsic pathway, also called mitochondrial-dependent apoptosis, is triggered by intracellular signals such as UPR, DNA damage, hypoxia, and viral infection. The main actors of the intrinsic pathway are proteins of the Bcl2 family, which include subfamilies of antiapoptotic, pro-apoptotic, and BH3-only proteins. In response to stress signals, members of the BH3-only proteins are activated and stimulate the assembly of pro-apoptotic effector, notably BAX and BAK into oligomers. These oligomers form a pore into the mitochondrial membrane that leads to the release of apoptotic factors into the cytosol, in particular cytochrome *C*. The cytochrome *C* associates within the apoptosome, a multiprotein complex, and initiates apoptosis via the recruitment of the inactive initiator caspase-9. Caspase 9 cleaves and activates effector caspases, caspase 3, and caspase 7, leading to apoptosis. This cascade can be alternatively activated through the upstream caspase-8 in response to an extrinsic signal (Kroemer et al. 2007).

Many viral proteins disturb normal cell physiology and deliver upstream signals that end up in a death response by apoptosis. Apoptosis is an integral part of the host defence against invading intracellular pathogens, in particular viruses, which serves to limit pathogen replication (Upton and Chan 2014; Li and Stollar 2004). However, viral genomes often encode apoptosis inhibitors in order to impair apoptosis and as such promote their replication and persistence (Everett and McFadden 1999). On the contrary, viruses can use apoptosis to kill infected host cells at the end of the viral replication cycle to increase the dissemination of their progeny and limit inflammatory responses. Due to the packing of the entire cellular content into apoptotic bodies, viruses or viral material can be rapidly taken up by surrounding cells (Kepp et al. 2009).

As CHIKV is highly cytopathic for mammalian cells, numerous studies have been conducted to define the type of cell death responsible for the cytopathic effect in CHIKV-infected cells.

CHIKV Activates Apoptosis

In vitro studies have shown that death of human infected cells is associated with the presence of a marker of apoptosis: active cleaved form of caspase-3 (Sourisseau et al. 2007). CHIKV-infected cells display a mitochondrial relocalisation of Bax, as well as the presence of cleaved PARP in infected cells, a well-known target of the effector caspases. It has also been shown, by using pharmacological inhibitors of apoptosis, as well as cells unable to engage the apoptotic pathway, that the main form of CHIKV-induced cell death is caspase-mediated apoptosis (Joubert et al. 2012; Krejbich-Trotot et al. 2011). To define whether the intrinsic or extrinsic

pathways are triggered upon CHIKV infection, the cleavage of two specific caspases, caspase-9 (intrinsic pathway) and caspase-8 (extrinsic pathway), has been analysed. CHIKV-induced apoptosis is triggered through an early caspase-9 intrinsic pathway, followed by a caspase-8 extrinsic dependent pathway. Moreover, CHIKV-induced apoptosis requires viral replication, as UV-inactivated CHIKV fails to cause apoptosis (Joubert et al. 2012; Krejbich-Trotot et al. 2011).

Pro-Viral Function of Apoptosis

Krejbich-Trotot et al. have reported that the apoptotic process promotes CHIKV dissemination in human cells (Krejbich-Trotot et al. 2011). They demonstrated that apoptosis inhibition decreases CHIKV infection by using drugs preventing apoptosis cell fragmentation, and that apoptosis contributes to perpetuate virus spreading through the formation of apoptotic bodies. Actually, CHIKV hijacks the apoptotic process through the formation and release of apoptotic blebs enclosing viral materials protected into membrane vesicles, promoting the infection of neighbouring cells (Fig. 3).

This mechanism was first reported for the SINV (Rosen et al. 1995). This process also limits the inflammatory response and thereby favours infection spreading in the infected host. Viral particles or materials enclosed within apoptotic vesicles are also protected from inactivation by host antibodies and proteases.

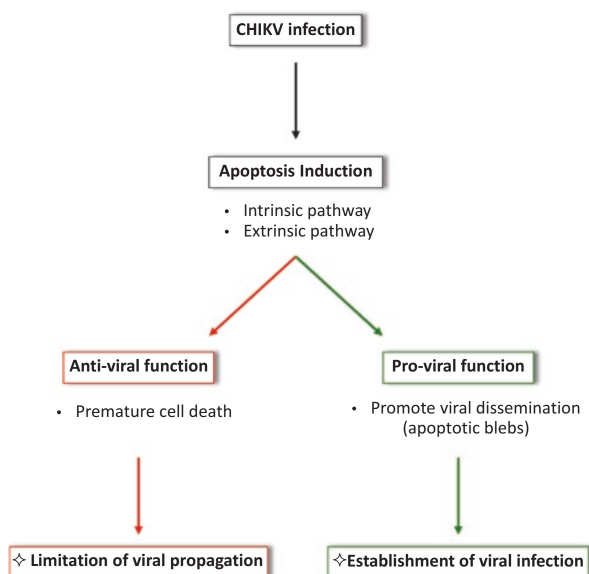

Fig. 3 Dual effect of apoptosis on CHIKV infection. CHIKV infection induces two apoptotic pathways, the intrinsic and extrinsic pathway. This induction of apoptosis can play either a pro- or an antiviral function. Apoptosis plays an antiviral role by promoting cell death limiting viral propagation. By forming apoptotic blebs containing viral components, apoptosis plays a pro-viral role. The apoptotic blebs disseminate the infection by infecting the neighbouring cells

Overall Effects of Autophagy and Apoptosis on Cell Survival and Infection

CHIKV, by subverting the autophagy machinery, protects human infected cells against cell death and favours its replication (Munz 2013). Cell death is essential in many biological processes, and apart from apoptosis, there is an increased recognised role of other death modalities such as necroptosis and autophagic cell death in host response to infection (Tait et al. 2014).

Joubert et al. have shown, in CHIKV-infected mouse cells, a relationship between autophagy and apoptosis. By using cells unable to engage either the autophagy or the apoptotic pathway, they provided evidence that autophagy in CHIKV-infected cells promotes cell survival and delays apoptosis upon infection (Joubert et al. 2012). Moreover, mice with reduced autophagy, Atg16L^{HM} mice (Cadwell et al. 2008), display higher susceptibility and higher lethality to CHIKV infection (Joubert et al. 2012). In human cells, the depletion of canonical mediators of autophagy, Beclin1 and Atg7, increases virus-induced cell death, indicating that autophagy also plays essentially a pro-survival role upon CHIKV infection in human cells (Judith et al. 2013; Fig.2). Two other autophagy mediators, p62 and NDP52, play a pro-survival role in CHIKV-infected human cells: p62 facilitates the clearance of CHIKV capsid, whereas NDP52 binds to CHIKV nsP2 in the cytosol and restricts transcriptional shutoff and apoptosis. Nuclear nsP2 indeed serves as a trigger for transcriptional shutoff and induction of apoptosis in SINV- and CHIKV-infected cells and these functions are assigned to its carboxy-terminal domain (Garmashova et al. 2006, 2007; Bourai et al. 2012). Thus, binding to NDP52 in the TGN-derived membranes retains nsP2 in the cytoplasm and restricts its migration in the nucleus, limiting transcriptional shutoff and cell death (Judith et al. 2013).

By facilitating the clearance of CHIKV capsid, autophagy plays an antiviral role, and limits infection-associated cell death. However, the cytoprotective role of autophagy, in addition to the fact that it is beneficial for the cell, can also be advantageous at the host level for the virus, as viral replication requires a living host cell. Premature cell death has also been considered as an anti-viral host mechanism that limits viral propagation (Fig. 3).

In conclusion, studies on CHIKV replication and the discovery that autophagy and apoptosis pathways are triggered by infection illustrate the intimate interconnection between these pathways in host response to infection.

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Chikungunya Virus Pathogenesis

David W. Hawman and Thomas E. Morrison

Chikungunya Virus Disease in Adults

Chikungunya virus (CHIKV) is notable in that the majority of those infected (72–96%) will develop symptoms (Sissoko et al. 2008; Moro et al. 2010; Gerardin et al. 2008b; Kumar et al. 2011; Queyriaux et al. 2008; Ayu et al. 2010). The mean time between infection and the development of symptoms (i.e., the incubation period) is typically 3 days (Rudolph et al. 2014). CHIKV is rarely fatal (Pialoux et al. 2007), however, neonates, the elderly, and individuals with underlying health conditions are the most likely to exhibit severe, atypical manifestations of infection, including death.

The most common clinical features of CHIKV infection are fever, polyarthralgia, myalgia, headache, and rash (Win et al. 2010; Ng et al. 2009; Simon et al. 2007; Manimunda et al. 2010; Sissoko et al. 2009; Moro et al. 2012; Rezza et al. 2007; Sharp et al. 2014). Less common clinical features include neurological involvement and hemorrhagic manifestations (Borgherini et al. 2007). Patients can exhibit high viral loads in the blood (>10^9 viral genomes/mL) (Panning et al. 2008), allowing for human–to–mosquito–to–human transmission cycles. Viremia is typically controlled by 7 days post-infection coincident with the detection of anti-CHIKV IgG antibodies (Kam et al. 2012a, b, c; Panning et al. 2008).

Reflecting the meaning of *chikungunya* in the Makonde language, “that which bends up,” one of the most notable clinical signs of CHIKV infection is severe joint pain. CHIKV-associated pain has been reported for most joints with the knee (Mathew et al. 2011; Manimunda et al. 2010) and small peripheral joints of the arms and legs most commonly affected (Simon et al. 2007). The pain is often bilateral and symmetric with involvement of multiple joints (Manimunda et al. 2010; Simon et al. 2007; Sissoko et al. 2009). Tenosynovitis, an inflammation of the linings of the

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tendons, is also seen in patients, with tendons of the wrists, fingers, and lower leg most frequently involved (Simon et al. 2007; Manimunda et al. 2010). X-ray and magnetic resonance imaging of joints of CHIKV-infected patients revealed joint effusion, bony erosion, marrow edema, synovial thickening, and tendonitis (Manimunda et al. 2010).

Chronic Chikungunya Virus Disease

A notable feature of CHIKV infection is that many patients develop chronic musculoskeletal symptoms, with wrist, ankle, knee, and joints of the hands most commonly affected. The development of prolonged symptoms is not unique to the recent re-emergence of CHIKV from Africa. A report from Fourie and Morrison describing a 1977 outbreak of CHIKV in South Africa noted episodic polyarthritis in some patients 18 months after acute infection (Fourie and Morrison 1979). In addition, a retrospective study of 107 patients from the same outbreak found that at 3 years after acute infection, 13 % of patients still reported joint pain (Brighton et al. 1983). However, the re-emergence of CHIKV during the past decade has provided an opportunity for clinical studies with larger patient cohorts and more detailed investigations that have refined our knowledge on the prevalence of chronic disease.

In one of the first reports on the 2005–2006 outbreak of CHIKV on multiple islands in the Indian Ocean region, 88 %, 86 %, and 48 % of patients remained symptomatic at 1, 3, and 6 months after disease onset, respectively (Simon et al. 2007). Similarly, a study examining a cohort of adult CHIKV patients during the 2005–2006 outbreak on Réunion Island reported that at a median of 1.5 years following acute infection ~64 % of patients reported persistent arthralgia (Borgherini et al. 2008). Of the patients reporting no persistent symptoms at the time of the study, the duration of arthralgia was ~3 months, suggesting that most of the adults infected with CHIKV during this outbreak exhibited symptoms lasting months to years following acute infection (Borgherini et al. 2008). A study by Sissoko et al. examining a cohort of patients also infected during the Réunion Island outbreak reported similar findings (Sissoko et al. 2009). At 15 months post-infection, 57 % of patients reported persistent rheumatological symptoms. The majority of patients (77 %) reported that initial symptoms lasted 15 days or longer, with 63 % reporting symptoms that lasted longer than 30 days (Sissoko et al. 2009). Similarly, 66.5 % of patients infected during the 2007 outbreak in Italy reported rheumatological symptoms after 12 months (Moro et al. 2012). Finally, a large study of 509 French travelers infected on Réunion Island found that the median duration of symptoms was 6–9 months, with 61 % of patients symptomatic at 1 year. Several studies have reported that the chronic symptoms associated with CHIKV infection may occur in a flaring or fluctuating manner (Couturier et al. 2012; Borgherini et al. 2008; Sissoko et al. 2009). In a more detailed examination (Couturier et al. 2012), relapses occurred on average 8 weeks apart with pain decreasing or variable but often occurring in the same joints.

Longer-term studies have found that CHIKV-associated rheumatological disease can last for years. For example, a study of French travelers infected on Réunion Island found that 59 % of individuals reported persistent arthralgia at 2 years post-acute infection (Larrieu et al. 2010). Similarly, a study on a cohort of Réunion Island patients revealed that >50 % of patients older than 45 still reported persistent joint pain 2 years after acute infection (Gerardin et al. 2013). Finally, a prospective study of patients, also on Réunion Island, found that 80 % of patients complained of arthralgia at 4 months post-illness onset with 60 % still exhibiting symptoms at 3 years (Schilte et al. 2013). These patients most commonly reported pain in the hands, wrists, ankles, and knees that usually involved multiple joints in a symmetrical manner. Clinical evaluation of the patients revealed that symptoms were associated with local swelling of the joint, weakness, and depression (Schilte et al. 2013).

Studies of recent CHIKV outbreaks outside of Réunion Island and other Indian Ocean islands have also reported the development of chronic disease. Outbreaks of CHIKV infection in 2008–2009 in Malaysia and Singapore both resulted in substantial chronic disease burdens. In Malaysia, the median duration of symptoms associated with CHIKV infection was 3 months, with 45 % of patients reporting arthralgia lasting longer than 4 months (Mohd Zim et al. 2013). In Singapore, 36 % of CHIKV patients remained symptomatic at 6 weeks (Win et al. 2010). Similarly, in a study of Indian patients following a CHIKV outbreak in 2008, 49.3 % of patients reported persistent symptoms at 10 months post-illness onset (Manimunda et al. 2010). Taken together, these studies clearly demonstrate that CHIKV infection is often associated with the development of chronic musculoskeletal disease involving pain and inflammation in numerous peripheral joints.

Chikungunya Virus Infection in Neonates

Perinatal mother-to-child transmission of CHIKV is associated with severe complications for the neonate, including fever, pain, prostration, thrombocytopenia, hemorrhagic disease, cardiac manifestations, and encephalitic disease characterized by brain swelling, hypotonia, coma, and seizures (Gerardin et al. 2008a; Ramful et al. 2007; Robillard et al. 2006b; Shrivastava et al. 2011). Furthermore, follow-up studies revealed that acute neurological involvement could result in global neurodevelopmental delay in coordination, language, sociability, and movement (Gerardin et al. 2014). In addition, atrophy of the frontal lobes has been reported (Gerardin et al. 2008a; Robin et al. 2008). Older children typically present with milder symptoms, although during the 2005–2006 outbreak on Réunion Island, two children aged 9.5 years rapidly succumbed to fatal CHIKV infection with clinical signs of neurological involvement (Robin et al. 2008). These clinical observations indicate that CHIKV infection in neonates can be severe, with a subset of these infants developing permanent disabilities. Similar to humans, CHIKV pathogenicity is strongly age-dependent in mice (Table 1), with young mice exhibiting enhanced susceptibility to CHIKV infection (Couderc et al. 2008; Werneke et al. 2011).

Table 1 Animal models of chikungunya virus infection

Models	Inoculation route	Major disease signs	Major tissues involved	Lethal	Chronicity	Notes	Reference
Nonhuman primates							
Adult Cynomolgus macaques	Intra-venous	Viremia, fever, rash, gingival bleeding, joint effusion,* edema,* encephalitis*	Spleen, lymph nodes, liver, joints, skin, meninges	Dose dependent	Yes,* persistent infectious virus in liver, spleen, muscle (44 dpi)	*Only seen with higher doses	Labadie et al. (2010)
Adult rhesus macaques	Intra-venous	Viremia, rash	NR	No	No	High dose	Messaoudi et al. (2013)
Aged rhesus macaques	Intra-venous	Viremia	NR	No	Yes, persistent RNA in spleen (35 dpi)	High dose	Messaoudi et al. (2013)
Pregnant rhesus macaques	Sub-cutaneous	Viremia, fever, joint swelling, leg swelling,* rash*	Spleen, lymph nodes, joints,* spinal cord,* liver	No	Yes, persistent RNA in spleen, lymph nodes, joints,* spinal cord* (21 dpi)	*Only seen in a subset of animals	Chen et al. (2010)
Mice							
Neonatal	Intra-dermal	Flacid paralysis	Muscle, joints, skin	Age dependent	NR	12-day-old mice resistant to lethal challenge	Couderc et al. (2008)
Neonatal outbred mice	Sub-cutaneous	Viremia, lethargy, altered gait, hind limb paralysis, muscle necrosis	Muscle, skin, brain	Yes, 8–17 %	NR		Ziegler et al. (2008)
3–4-week-old C57BL/6	Footpad	Viremia, footpad swelling, arthritis, tendonitis, tenosynovitis, myositis	Joints, spleen	No	Yes, chronic joint pathology and persistent viral RNA (112 dpi)		Morrison et al. (2011); Hawman et al. (2013)
Adult C57BL/6	Footpad	Viremia, footpad swelling, arthritis, tenosynovitis, myositis	Joints, spleen, lymph nodes, liver	No	Yes, chronic joint pathology and persistent viral RNA (100 dpi)		Gardner et al. (2010); Poo et al. (2014)
Adult IFN-α/βR$^{+/-}$	Intra-dermal	NR	Muscle, joints	No	No*	*As measured by infectious virus	Couderc et al. (2008)
Adult IFN-α/βR$^{-/-}$	Intra-dermal	Muscle weakness, lethargy	Muscle, joints, skin, brain, serum, liver, stomach	Yes, 100 %	NA	Rapidly fatal	Couderc et al. (2008)

*are only observed in animals that received a higher dose.

Risk Factors for Acute and Chronic CHIKV Disease

Risk factors for acute and chronic CHIKV disease have been identified. Increased age is associated with more severe disease and hospitalization (Borgherini et al. 2007; Tandale et al. 2009; Couturier et al. 2012), and young children and neonates are at increased risk for developing CNS disease (Gerardin et al. 2008a; Robillard et al. 2006a; Robin et al. 2008). In addition, multiple studies have reported increased age as a risk factor for developing chronic CHIKV disease symptoms (Essackjee et al. 2013; Gerardin et al. 2013; Hoarau et al. 2010; Couturier et al. 2012; Dupuis-Maguiraga et al. 2012; Mohd Zim et al. 2013; Moro et al. 2012; Schilte et al. 2013; Sissoko et al. 2009; Thiberville et al. 2013; Vijayakumar et al. 2011). The presence of comorbidities, such as hypertension and diabetes (Gerardin et al. 2013; Sissoko et al. 2009; Schilte et al. 2013; Yaseen et al. 2014), or pre-existing joint pain (Borgherini et al. 2008; Couturier et al. 2012; Dupuis-Maguiraga et al. 2012; Moro et al. 2012; Sissoko et al. 2009) have also been found to increase the risk for development of chronic CHIKV disease. Several studies have reported that women are more likely to report chronic musculoskeletal symptoms (Essackjee et al. 2013; Mathew et al. 2011; Win et al. 2010; Thiberville et al. 2013; Kularatne et al. 2012; Moro et al. 2012) possibly reflecting sex-based differences in immunity (Oertelt-Prigione 2012; Moro et al. 2012). However, some studies have failed to find a correlation between gender and resolution of disease (Mohd Zim et al. 2013; Couturier et al. 2012). Complicating the picture, the severity of acute CHIKV disease symptoms has been found to correlate positively (Larrieu et al. 2010; Dupuis-Maguiraga et al. 2012; Sissoko et al. 2009; Gerardin et al. 2013; Yaseen et al. 2014), correlate negatively (Kam et al. 2012c), or not correlate (Mohd Zim et al. 2013) with the development of chronic disease. Similarly, viral loads during the acute stage of CHIKV infection have been inconsistently associated with increased risk of chronic disease (Hoarau et al. 2010; Win et al. 2010). Thus, future work is required to understand fully the risk factors for acute and chronic CHIKV disease.

Acute and Chronic CHIKV Infection: Cellular and Tissue Targets

Natural CHIKV infection begins when an infected mosquito delivers the virus into the skin during a blood meal (Fig. 1). Studies suggest that the mosquito bite modulates the initial host response to CHIKV infection (Thangamani et al. 2010), however, little is known about the initial cellular sites of viral replication or the precise mechanisms by which the virus then disseminates within the host. In mice, analysis of CHIKV-infected cells at the inoculation site revealed vimentin-positive cells, a marker of fibroblasts, as the predominant cell type infected (Schilte et al. 2010; Couderc et al. 2008). After initial replication, CHIKV rapidly replicates and disseminates within the host. CHIKV-infected humans, nonhuman primates (NHPs),

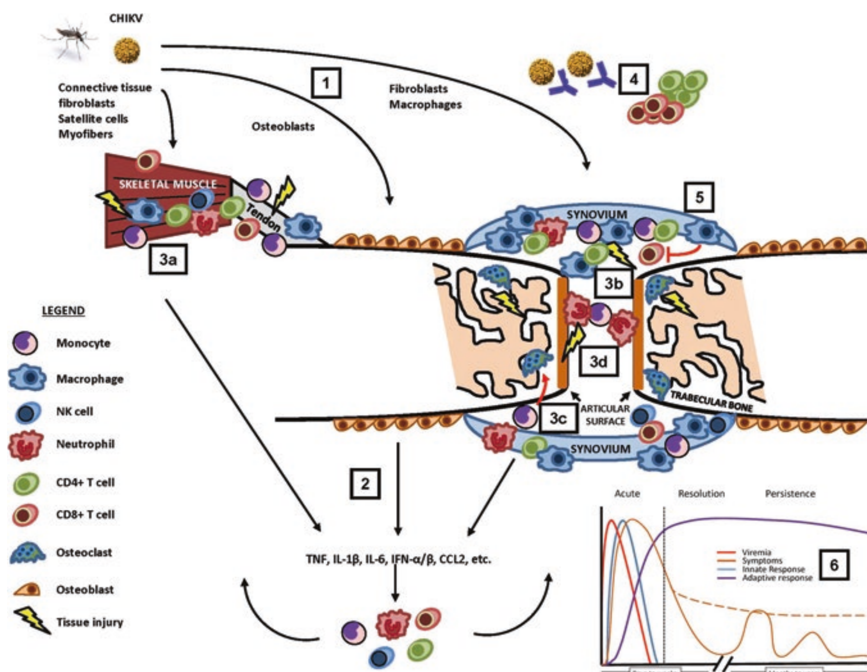

Fig. 1 *CHIKV infection, inflammation, and injury in musculoskeletal tissues*. [*1*] Following inoculation and local replication in the skin, CHIKV disseminates to musculoskeletal tissues including skeletal muscles, tendons, bones, and synovial membranes. Existing evidence suggests that the virus infects and replicates within connective tissue fibroblasts, satellite cells, myofibers, osteoblasts, and possibly macrophages within these tissues. [*2*] CHIKV infection and replication in cells in musculoskeletal tissues results in local production of inflammatory mediators that recruit and activate immune cells including monocytes, neutrophils, NK cells, and T cells. [*3*] In turn, tissue-infiltrating immune cells produce inflammatory mediators that promote recruitment of additional immune cells and contribute to tissue injury. For example, [*3a*] monocytes and monocyte-derived macrophages have been implicated in tissue injury in skeletal muscle tissue, [*3b*] $CD4^{+}$ T cells and macrophages have been implicated in joint tissue injury and edema, [*3c*] differentiation of recruited monocytes to osteoclasts, by factors released from infected osteoblasts, has been implicated in bone erosion, and *[3d]* in $CCR2^{-/-}$ and $TLR3^{-/-}$ mice, neutrophils have been implicated in damage to cartilage and other injury in joint-associated tissues. [*4*] In addition to activating a robust innate immune response that contributes to control of infection (particularly the type I IFN response), CHIKV induces both cell-mediated and humoral immune responses that contribute to control of infection. [*5*] An M2-like macrophage response, likely driven in response to tissue damage, may impair T-cell-mediated clearance of the virus. [*6*] Following the induction of adaptive immune responses, some patients will resolve the infection. However, a subset of patients will progress to chronic disease, characterized by continuous or flaring inflammation and pain in joints and tendons that may be due to persistent CHIKV infection in musculoskeletal tissues

and mice can exhibit viral titers from 10^{6}–10^{9} PFU/mL of blood (Win et al. 2010; Panning et al. 2008; Labadie et al. 2010; Gardner et al. 2010).

Human and animal studies have also demonstrated that CHIKV replicates within musculoskeletal tissues, suggesting that the musculoskeletal disease is initiated by direct viral replication in the affected tissues. At these sites, connective tissue

fibroblasts, osteoblasts, muscle cells, and possibly macrophages serve as the predominant targets of CHIKV infection (Fig. 1). In humans, CHIKV antigen has been detected in tissues including the muscle, skin, and joint (Ozden et al. 2007; Couderc et al. 2008). In addition, longer-term studies have detected CHIKV RNA and antigen in joint and muscle tissue weeks after the onset of symptoms (Ozden et al. 2007; Hoarau et al. 2010). Infection of NHPs with CHIKV shows parallels to human infection. Shortly after infection of cynomolgous macaques with CHIKV, high levels of infectious virus were detectable in the joint, muscle, liver, spleen, and lymph node (Labadie et al. 2010). Macaques also developed classical signs of human CHIKV infection including high viremia, fever, rash, and joint effusions (Labadie et al. 2010; Messaoudi et al. 2013). In a pregnant rhesus macaque model, animals exhibited viremia and joint symptoms along with detection of viral RNA in the spleen, lymph nodes, joint-associated connective tissue, and muscle (Chen et al. 2010; Table 1).

Similar to the human and NHP studies, CHIKV infection in mice shows a strong tropism for musculoskeletal tissues (Fig. 1). Mice inoculated with CHIKV have consistently shown high viral burdens in muscle and joint tissues (Gardner et al. 2010, 2014; Hawman et al. 2013; Morrison et al. 2011; Ziegler et al. 2008). Upon inoculation of the virus into the footpad, mice also exhibit significant swelling of the ipsilateral foot and ankle concordant with histological evidence of tissue damage including necrosis, arthritis, and tenosynovitis (Morrison et al. 2011; Gardner et al. 2010, 2014; Ziegler et al. 2008). The main target cells in joints appear to be fibroblasts. Similarly, the predominant target cells in skeletal muscle tissue appear to be fibroblasts of the epimysium and perimysium (Couderc et al. 2008). However, the ability of CHIKV to infect murine myofibers has been found to be associated with severe muscle necrosis and the development of more severe disease (Rohatgi et al. 2014). Microcomputed tomographic analysis of infected mice also revealed significant reduction of bone volume in the tibial epiphysis following CHIKV infection, which was associated with virus infection of bone-associated tissues (Chen et al. 2014a). These findings recapitulate observations of bone loss and damage in some CHIKV-infected patients (Manimunda et al. 2010; Malvy et al. 2009). Cumulatively, the data from both human studies and animal models of CHIKV infection demonstrate that the musculoskeletal pain and injury are likely initiated by direct CHIKV infection and replication in cells within the affected tissues.

Several studies have also detected CHIKV infection of the liver (Couderc et al. 2008; Gardner et al. 2010; Hawman et al. 2013; Chen et al. 2010), which may serve as an early site of viral amplification (Couderc et al. 2008). In addition, significant viral loads in lymphoid tissue have been detected (Gardner et al. 2010; Hawman et al. 2013; Couderc et al. 2008; Labadie et al. 2010; Chen et al. 2010; Messaoudi et al. 2013), possibly due to infection of macrophages (Couderc et al. 2008; Gardner et al. 2010). However, the consequence of CHIKV infection of these tissues are not well understood.

Animal models have also shown that an immature or impaired immune response can lead to significant viral burdens in the CNS. Newborn or IFNα/βR$^{-/-}$ mice develop high viral loads in CNS tissues and rapidly succumb to CHIKV infection (Couderc et al. 2008; Gorchakov et al. 2012; Rohatgi et al. 2014; Ziegler et al. 2008). Two-to-four-week old mice by contrast showed early detection of virus in the

CNS but were able to resolve the infection successfully (Hawman et al. 2013; Ziegler et al. 2008). In addition, macaques receiving high doses of CHIKV developed a high-titer viremia along with signs of meningoencephalitis and, in some cases, mortality (Labadie et al. 2010). Together, these models recapitulate many of the atypical outcomes of CHIKV infection observed in patients with compromised or immature immune systems.

As discussed above, the development of chronic joint pain in a subset of individuals is a central feature of infection with CHIKV. The cause of this persistent joint disease is unclear, however, an increasing body of evidence suggests that CHIKV may establish chronic infections in joint-associated tissues.

In a limited number of human studies, CHIKV antigen and RNA have been detected in synovial tissue biopsies collected from patients suffering from chronic joint pain, with CHIKV antigen detected in perivascular macrophages (Hoarau et al. 2010). In addition, CHIKV antigen was detected in muscle satellite cells in muscle biopsy tissue collected from a patient during a relapse of chronic musculoskeletal pain (Ozden et al. 2007). Similar to these findings, Ross River virus RNA has been detected in knee biopsies collected 5 weeks after the onset of joint symptoms (Soden et al. 2000).

Experiments in animal models further suggest that CHIKV may establish chronic infections in musculoskeletal and other tissues. CHIKV RNA and antigens were detected up to 90 days post-inoculation in the spleen, lymph nodes, liver, and, to a lesser extent, in synovial and muscle tissue of infected cynomolgus macaques (Labadie et al. 2010). Similarly, CHIKV RNA was found to persist in the spleen of experimentally infected aged rhesus macaques (Messaoudi et al. 2013). In mice, infection with a recombinant CHIKV expressing luciferase resulted in detection of luciferase activity in joint-associated tissues near the site of inoculation out to 60 days post-infection (Teo et al. 2013). Finally, CHIKV RNA was shown to persist for months in joint-associated tissues of infected wild-type mice, but not a variety of other tissues; and the persistence of CHIKV RNA in joints was associated with chronic synovitis (Hawman et al. 2013).

Viral Determinants of CHIKV Pathogenesis

Viral determinants of CHIKV pathogenesis in humans are not well defined. Since discovery of CHIKV in the 1950s during an outbreak of febrile arthritis (Robinson 1955), phylogenetic studies have defined three genotypes of the virus: West African, East Central South African (ECSA), and Asian (Powers et al. 2000). Among the three genotypes, virus genomic sequences differ at the nucleotide level by up to 15 %. However, human disease does not appear to be unique to infection with CHIKV strains from a particular genotype, with epidemics involving viruses from each genotype resulting in both acute and chronic musculoskeletal disease (Sharp et al. 2014; Ayu et al. 2010; Schuffenecker et al. 2006; Thonnon et al. 1999; Powers and Logue 2007). Recapitulating these observations, CHIKV infection of animal

models has shown that musculoskeletal disease develops following infection with strains representative of all three genotypes. However, some differences between strains have been observed. In mice, CHIKV of the ECSA lineage was found to cause more severe disease than Asian or West African lineage viruses (Gardner et al. 2010; Rohatgi et al. 2014). In macaques, ECSA and West African lineage viruses caused similar viremia (Chen et al. 2010; Messaoudi et al. 2013), but ECSA viruses induced a more potent T-cell response (Messaoudi et al. 2013). CHIKV strains from all genotypes have been shown to persist long-term in mice (Hawman et al. 2013) and in macaques (Chen et al. 2010; Messaoudi et al. 2013), further suggesting that prolonged musculoskeletal disease is common to infection with CHIKV strains from all genotypes.

Studies investigating deliberately attenuated strains of CHIKV as vaccine candidates have revealed that residues in the CHIKV E2 glycoprotein can alter pathogenesis. Much of this work was initiated based on the observation that the CHIKV vaccine strain 181/25, generated by serial plaque passages in MRC-5 human lung fibroblasts, exhibited attenuated disease outcomes in humans and in mice (Levitt et al. 1986; Edelman et al. 2000). Studies to define the attenuating mutations revealed that two mutations in E2 were sufficient to attenuate the virus, likely by enhancing viral interactions with glycosaminoglycans (GAGs) and inhibiting the ability of the virus to disseminate within the host (Ashbrook et al. 2014; Silva et al. 2014; Gorchakov et al. 2012; Gardner et al. 2012, 2014). Consistent with these data, additional mutations of surface-exposed residues of E2 that increase electrostatic potential were identified in cell-culture passaged CHIKV and shown to attenuate CHIKV-induced musculoskeletal disease in mice (Gardner et al. 2014).

Much less is known about specific virulence determinants in other coding and noncoding regions of the CHIKV genome. In vitro experiments suggest that the nonstructural proteins nsP2 and nsP3 play important roles in suppressing antiviral responses (Fros et al. 2010, 2012; Akhrymuk et al. 2012). Additionally, the fidelity of the nsP4 RNA-dependent RNA-polymerase (RdRp) is also a determinant of CHIKV pathogenesis, as mutations that increase or decrease RdRp fidelity result in attenuation in vivo (Coffey et al. 2011; Rozen-Gagnon et al. 2014). However, our understanding of the viral determinants of CHIKV pathogenesis remains limited.

Cellular Mediators of CHIKV Pathogenesis

Data from human CHIKV infections suggest that the pain in musculoskeletal tissues reported by patients may be due to immune cell infiltration and injury of these tissues (Fig. 1). A muscle biopsy of a CHIKV-infected patient during acute disease revealed atrophy and necrosis of muscle fibers along with infiltration by immune cells (Ozden et al. 2007). A muscle biopsy from a separate patient suffering musculoskeletal symptoms weeks after acute infection also showed extensive immune cell infiltration (Ozden et al. 2007). In addition, T cells, macrophages, and natural killer (NK) cells were detected in synovial fluid and tissue collected from a CHIKV

patient with chronic arthralgia (Hoarau et al. 2010). Similarly, joint tissue biopsies from patients infected with Ross River virus revealed mononuclear cell infiltration (Soden et al. 2000). Together, these data suggest that following infection, CHIKV disease involves infiltration of musculoskeletal tissues by immune cells.

These observations in human patients are supported by studies in animal models that show infection and inflammation of joint-associated tissue following CHIKV infection (Fig. 1). C57BL/6 mice inoculated with CHIKV show histopathological evidence of arthritis, tenosynovitis, and myositis, with infiltration of the tissues by monocytes, macrophages, NK cells, and T lymphocytes (Gardner et al. 2010; Morrison et al. 2011). Ross River virus infection of mice also results in infiltration of joint and skeletal muscle tissues, with monocytes, NK cells, and T cells comprising the major cellular infiltrates (Morrison et al. 2006). The infiltration of these tissues with immune cells likely reflects the strong tropism that CHIKV and Ross River virus exhibit for connective tissue fibroblasts, myofibers, cells of the synovium and periosteum, and muscle satellite cells (Morrison et al. 2006; Sourisseau et al. 2007; Ozden et al. 2007; Schilte et al. 2010; Rohatgi et al. 2014; Chen et al. 2014b). Thus, although CHIKV is cytopathic in vitro and may contribute directly to cellular death and tissue damage in vivo, the coincidence of infiltrating immune cells with major tissue pathology and pain suggests a possible immunopathogenic component of CHIKV disease.

Myeloid Cells

A number of studies have implicated various myeloid cell populations in CHIKV pathogenesis. As discussed above, monocytes have been detected in synovial fluid collected from a patient with chronic CHIKV disease, and macrophages were present in both synovial fluid and the synovium (Hoarau et al. 2010). These findings are similar to previous studies showing that the cellular infiltrate in the synovial fluid of patients with Ross River virus disease is composed predominantly of monocytes and macrophages (Fraser et al. 1981). The roles of these myeloid cells, as well as neutrophils and dendritic cells, in CHIKV pathogenesis has been an area of intense research in animal models. Treatment of mice with clodronate-loaded liposomes, which deplete phagocytic cells including monocytes and macrophages, reduced the severity of acute disease signs in both CHIKV- and Ross River virus-infected mice (Gardner et al. 2010; Lidbury et al. 2008), suggesting a pathogenic role for these cell types during these infections (Fig. 1). More recent studies in mice suggest that monocytes recruited to musculoskeletal tissues during CHIKV or Ross River virus infection can be differentiated to osteoclasts that mediate focal bone erosion (Chen et al. 2014a, b), a feature of CHIKV infection that has been observed in some patients (Malvy et al. 2009; Manimunda et al. 2010; Fig. 1). However, mice deficient in CCR2, a chemokine receptor that regulates trafficking and recruitment of monocytes, basophils, and T cells, developed significantly more severe and prolonged CHIKV-induced arthritis (Poo et al. 2014). In CHIKV-infected CCR2$^{-/-}$ mice, the

number of monocytes and macrophages in joint tissues was substantially reduced whereas the number of neutrophils, as well as cartilage damage, was dramatically increased (Fig. 1). These findings suggest that recruitment of monocytes to sites of infection may also protect from more severe, neutrophil-mediated, joint injury during CHIKV infection. Neutrophils have also been implicated in the more severe tissue injury observed in musculoskeletal tissues of CHIKV-infected TLR3$^{-/-}$ mice (Her et al. 2014). Thus, further studies are required to elucidate the precise role of monocyte, macrophage, and neutrophil cell populations in CHIKV pathogenesis. Finally, although relatively understudied to date, dendritic cells may also directly regulate CHIKV-induced arthritic disease. In mice, dendritic cells have been detected in joint-associated tissues of the foot and ankle (Long et al. 2013). During CHIKV infection, dendritic cells in these tissues were shown to have reduced expression of the dendritic cell immunoreceptor (DCIR), a C-type lectin receptor implicated in regulation of inflammatory responses. CHIKV infection of DCIR$^{-/-}$ mice resulted in more rapid and more severe arthritic disease, suggesting that DCIR-expressing dendritic cells may limit CHIKV-induced tissue inflammation and injury.

NK Cells

The role of NK cells in CHIKV pathogenesis is not well understood and has not been extensively investigated in animal models. However, studies of human CHIKV infection suggest that NK cell responses may be important determinants of the outcome of infection. During acute CHIKV infection, NK cells in circulation are strongly activated (Petitdemange et al. 2011; Hoarau et al. 2010), and activated NK cells have been detected in synovial tissue during the chronic stage (Hoarau et al. 2010). NK cells are regulated by a combination of activating and inhibitory receptors, which include the killer immunoglobulin-like receptor (KIR) family that interacts with MHC class I molecules (Orr and Lanier 2010). During acute CHIKV infection, expansion of NK cells that coexpress CD94/NKG2C and the KIR family members KIR2DL2/DL3 was positively correlated with viral load, whereas expression of KIR2DL1 was inversely associated with viral load (Petitdemange et al. 2011). Although the NK cells expanded during CHIKV infection were found to be impaired for IFN-γ production, these cells were shown to have strong cytolytic capacity, suggesting that CHIKV infection activates a subset of NK cells that may be important for the clearance of CHIKV-infected cells. In a related study, the KIR genotype of CHIKV-infected patients was found to differ significantly from that in healthy controls or dengue virus-infected patients (Petitdemange et al. 2014), further suggesting that the NK cell KIR repertoire may contribute to susceptibility to CHIKV infection. This study also reported an increase in the frequency of HLA-C2, a ligand for KIR2DL1, in combination with KIR2DL1 in CHIKV-infected patients. These data, in combination with the data demonstrating a depletion of KIR2DL1-positive cells during acute infection (discussed above), support a role for KIR2DL1-HLA-C2 interactions in CHIKV pathogenesis.

T Lymphocytes

During acute CHIKV infection in humans and macaques, an increase in activated peripheral T cells has been reported (Wauquier et al. 2011; Hoarau et al. 2010; Messaoudi et al. 2013). $CD4^+$ and $CD8^+$ T cells from CHIKV-infected patients and macaques produced IFN-γ in response to CHIKV antigens including E2, E1, capsid, and nsP1, with responses against E2 being the highest in magnitude (Hoarau et al. 2013; Messaoudi et al. 2013). These data indicate that CHIKV infection results in activation of virus antigen-specific T cells, however, the roles of these cells in pathogenesis are still being elucidated. In comparison to adult macaques, the magnitude and the breadth of CHIKV-specific T-cell responses were reduced in aged macaques (Messaoudi et al. 2013). The reduced T-cell responses in aged macaques were associated with enhanced CHIKV persistence (Messaoudi et al. 2013), suggesting that T cells contribute to the clearance of CHIKV infection and protect against the development of viral persistence (Fig. 1). Human CHIKV infection is also associated with T-cell infiltration of musculoskeletal tissues. T cells have been detected in synovial fluid as well as synovial and muscle tissue biopsies collected from CHIKV-infected patients (Hoarau et al. 2010; Ozden et al. 2007). Consistent with these findings, studies in both CHIKV- and Ross River virus-infected mice have shown that $CD4^+$ and $CD8^+$ T cells infiltrate musculoskeletal tissues (Morrison et al. 2006, 2007, 2011; Gardner et al. 2010). Infiltrating T cells also likely contribute to pathology as mice deficient in *Rag1*, which lack mature B and T cells, showed improved tissue pathology relative to WT mice during acute CHIKV infection (Hawman et al. 2013). Furthermore, $CD4^{-/-}$ mice and WT mice depleted of $CD4^+$ T cells showed significantly reduced foot pad swelling and joint pathology, suggesting a pathological role for $CD4^+$ T cells in CHIKV infection (Teo et al. 2013; Her et al. 2014; Fig. 1). $CD4^+$ T cells mediated enhanced joint pathology even in the absence of IFN-γ, suggesting that these cells exacerbate disease via an IFN-γ-independent mechanism (Teo et al. 2013). However, a separate study reported that genetic deletion of IFN-γ reduced CHIKV-induced joint swelling in mice (Nakaya et al. 2012), thus, the mechanism(s) by which $CD4^+$ T cells contribute to CHIKV disease require further investigation.

B Lymphocytes

In humans, CHIKV-specific IgM and IgG become detectable during the acute phase (~5 to 10 days post-illness onset) (Kam et al. 2012c), indicating that B cells are activated at early stages of infection. Similarly, CHIKV-specific antibodies are detectable as soon as 2 days post-infection in mice (Lum et al. 2013) and as early as day 10 in NHPs (Messaoudi et al. 2013). Anti-CHIKV antibodies from humans and animal models are neutralizing (Lum et al. 2013; Kam et al. 2012c, 2014) and contribute to clearance, as μMT mice lacking B-cell responses sustain persistent viremia (Lum et al. 2013). In addition, neutralizing antibodies administered prior to or shortly after infection can protect against lethal disease in mice (Goh et al. 2013; Pal

et al. 2013, 2014; Couderc et al. 2009; Fric et al. 2013) and limit viral dissemination in NHPs (Pal et al. 2014).

In studies in both humans and animal models, the magnitude and quality of the B-cell response has been shown to affect CHIKV pathogenesis. CHIKV-specific IgG3 was reported as the dominant human isotype, and its accumulation was associated with more efficient virus clearance (Kam et al. 2012c). In macaques, reduced B-cell responses in aged animals were also associated with enhanced CHIKV persistence (Messaoudi et al. 2013). Finally, recent studies in mice suggest that poor-quality antibody responses in TLR3$^{-/-}$ mice contribute to the enhanced CHIKV replication and tissue injury observed in these animals (Her et al. 2014). These findings are consistent with previous studies in a mouse model of Ross River virus infection which showed that the more severe Ross River disease observed in TLR7$^{-/-}$ mice was associated with high levels of poorly neutralizing antibodies and less efficient virus clearance (Neighbours et al. 2012).

Cytokines and Chemokines in CHIKV Pathogenesis

Type I IFN

Increasing evidence suggests that specific cytokines and chemokines influence the pathogenesis of CHIKV infection. High levels of IFN-α were detected in the serum of CHIKV-infected infants and adults (Schilte et al. 2010; Werneke et al. 2011) and the expression of numerous interferon-stimulated genes (ISGs) was elevated in PBMCs of CHIKV-infected patients (Teng et al. 2012). These findings indicate that CHIKV infection results in the production of type I IFN and activation of downstream signaling and gene expression.

The production of type I IFN, type I IFN signaling, and downstream effectors of the type I IFN response are critical determinants of CHIKV pathogenesis, as abundant data from animal models indicate that a variety of specific deficits in the type I interferon pathway lead to more severe disease manifestations. CHIKV infection of mice deficient in the receptor for type I IFN (*Ifnar1*$^{-/-}$) results in more severe disease, with mice succumbing to the infection within 2–4 days (Schilte et al. 2010; Couderc et al. 2008). Similarly, mice deficient in the transcription factor STAT1, which is activated downstream of the type I IFN receptor, develop more severe signs of joint disease and succumb to CHIKV infection (Schilte et al. 2010; Gardner et al. 2012). Together, these findings indicate that the ability to respond to type I IFN regulates CHIKV pathogenesis.

The production of type I IFN is triggered by the interaction of pathogen-associated molecular patterns (PAMPs) with host pattern recognition receptors (PRRs) including Toll-like receptors (TLRs) and RNA helicases such as RIG-I and MDA-5. In contrast to Ifnar1$^{-/-}$ and STAT1$^{-/-}$ mice, RIG-1$^{-/-}$, MDA-5$^{-/-}$, MAVS$^{-/-}$, TRIF$^{-/-}$, and MyD88$^{-/-}$ mice (molecules involved in host sensing of RNA virus infection) all survive CHIKV infection (Schilte et al. 2010; Rudd et al. 2012), suggesting that multiple host detection pathways that lead to the production of type I IFN contribute to the control of

CHIKV infection in vivo. Thus, genetic deletion of a single sensor pathway is not sufficient to result in enhanced mortality. However, CHIKV-induced foot swelling was found to be more severe in TRIF$^{-/-}$ and MAVS$^{-/-}$ mice (Rudd et al. 2012), suggesting that RIG-I/MDA-5 signaling through MAVS, and TLR3 signaling via TRIF, control the severity of CHIKV-induced arthritis. Consistent with these data, genetic deletion of TLR3 in mice results in enhanced CHIKV dissemination and more severe pathology in joint-associated tissues (Her et al. 2014). Complementing the studies in mice, a polymorphism in the human *TLR3* gene was found to be associated with increased CHIKV disease severity (Her et al. 2014), further supporting an important role for TLR3 in control of CHIKV infection and pathogenesis.

IRF-3 and IRF-7 are transcription factors activated downstream of PRRs that regulate the induction of IFN-β and IFN-α genes. Although IRF3$^{-/-}$ mice and IRF7$^{-/-}$ mice survive CHIKV infection, both strains exhibit more severe signs of joint disease (Rudd et al. 2012; Schilte et al. 2012). Furthermore, IRF3/7$^{-/-}$ mice exhibit signs of severe joint disease, dramatically elevated viral loads in tissues, and succumb to CHIKV infection (Rudd et al. 2012; Schilte et al. 2012). The mortality in CHIKV-infected IRF3/7$^{-/-}$ mice was associated with severe edema and hemorrhage (Rudd et al. 2012), indicating a critical role for these transcription factors in control of the severity of CHIKV-induced disease.

Other Cytokines and Chemokines

Acute and chronic CHIKV disease in humans is associated with elevated levels of specific cytokines and chemokines in the circulation. Elevated levels of IFN-γ, IL-6, IL-1β, macrophage migration inhibitor factor (MIF), CCL2 (MCP-1), CXCL9 (MIG), and CXCL10 (IP-10) have been detected in patients with acute CHIKV infection, with levels of IL-6, IL-1β, RANTES, MCP-1, MIG, and IP-10 associated with increased disease severity (Lohachanakul et al. 2012; Ng et al. 2009; Kelvin et al. 2011; Venugopalan et al. 2014; Herrero et al. 2013).

Cytokines and chemokines may also contribute to chronic CHIKV disease. In some studies, persistent arthralgia has been associated with elevated levels of IL-6, GM-CSF, MIG, and MCP-1 (Chow et al. 2011; Reddy et al. 2014). Complementing these data, the serum levels of MCP-1, MIG, and IP-10 correlated with the degree of joint swelling in CHIKV-infected mice (Gardner et al. 2014). Furthermore, treatment of mice with bindarit, an inhibitor of MCP-1 synthesis, reduced the severity of CHIKV- and Ross River virus-induced musculoskeletal tissue inflammation and injury (Rulli et al. 2009, 2011), suggesting that MCP-1-mediated recruitment of monocytes contributes to disease severity. Finally, although viral loads were unaffected, the expression of numerous proinflammatory cytokines and chemokines were increased in neonatal ISG15$^{-/-}$ mice, which are highly susceptible to CHIKV infection (Werneke et al. 2011), providing further evidence that cytokines and chemokines contribute to the pathogenicity of CHIKV.

Complement

Activation of the complement system plays an important role in the pathogenesis of rheumatoid arthritis (Holers 2014). The role of the complement pathway in CHIKV-induced arthritis has not been extensively investigated, however, recent gene expression analyses in mice suggest that complement is activated during CHIKV infection (Nakaya et al. 2012). Furthermore, activated complement was detected in the synovial fluid of Ross River virus-infected patients (Morrison et al. 2007), and the levels of mannose binding lectin (MBL), an activator of the complement cascade, in the serum and synovial fluid of patients with Ross River virus infection correlated with disease severity (Gunn et al. 2012). Complement was also shown to play a pathologic role in Ross River virus-induced disease in mice, with $C3^{-/-}$, $CR3^{-/-}$, and $MBL^{-/-}$ mice all developing less severe musculoskeletal tissue damage and disease signs (Gunn et al. 2012; Morrison et al. 2007, 2008).

Summary

Although great strides have been made in recent years, the molecular mechanisms that contribute to CHIKV pathogenesis, particularly in humans, are not well understood. Our knowledge of the viral determinants of disease is limited, and many viral mechanisms that contribute to disease remain to be elucidated. The antiviral immune response against CHIKV infection appears to be robust, with strong induction of interferon as well as virus-specific antibody and T-cell responses. Studies in humans and animal models indicate that these responses are necessary to control viral replication and dissemination, and to resolve the infection. However, studies also suggest a pathogenic component to the immune response elicited during CHIKV infection. Further studies in animal models are required to define the precise immune and inflammatory mediators of protection and pathology, and then to integrate these findings with studies of human disease. Finally, despite the seemingly rapid and robust anti-CHIKV immune response, many of those infected develop chronic musculoskeletal pain that can last for years. Our understanding of the host and viral mechanisms that contribute to the development of chronic CHIKV disease is limited. However, data from multiple animal models, and limited data from human samples, suggest that chronic disease may be due to chronic CHIKV infection. The possibility that CHIKV establishes long-term infection raises a variety of intriguing questions regarding the virological and immunological mechanisms that contribute to chronic disease. Advancing our understanding of acute and chronic CHIKV pathogenesis is warranted given the continued geographic expansion of CHIKV outbreaks, and the lack of approved vaccines and therapeutic options for preventing or treating CHIKV infection.

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Restriction Factors and Chikungunya Virus

Wadie D. Mahauad-Fernandez and Chioma M. Okeoma

Introduction

Host-encoded viral restriction factors are part of the cell-intrinsic defenses of the innate immune system, also called intrinsic immunity. Contrary to other cell-based antiviral responses that require de novo gene activation, expression, and protein translation, restriction factors are constitutively expressed in most cell types and they act to prevent the infection and spread of viruses. To be classified as a restriction factor, a protein has to meet four criteria including, (1) the ability to restrict virus replication, (2) susceptibility to neutralization by viral encoded factor(s), (3) the presence of signatures of positive selection, and (4) the ability to respond to interferon (IFN) signaling (Harris et al. 2012).

Thus far, a handful of restriction factors have been discovered to impede retroviral infection. These include apolipoprotein B mRNA editing enzyme, catalytic polypeptide 3 (APOBEC3), bone marrow stromal antigen 2 (BST-2/tetherin), MX dynamin-like GTPase 2 (MxB), SAM domain and HD domain 1 (SAMHD1), Schlafen 11 (SLFN11), and Tripartite motif protein 5α (TRIM-5α). Of these, only BST-2/tetherin has been implicated in CHIKV pathogenesis at the time of this report. Here, we discuss available data on BST-2-mediated restriction of CHIKV infection of host cells and the release of nascent CHIKV particles from host cells. Also discussed is the regulation of BST-2 by CHIKV.

BST-2 is an IFN-inducible type II transmembrane protein (Ishikawa et al. 1995) mainly located on the cell membrane but also present in intracellular compartments, such as the endoplasmic reticulum (ER), trans-Golgi apparatus (TGN), and early endosomes (Hotter et al. 2013; Hammonds et al. 2012; Habermann et al. 2010; Fujita et al. 2012).

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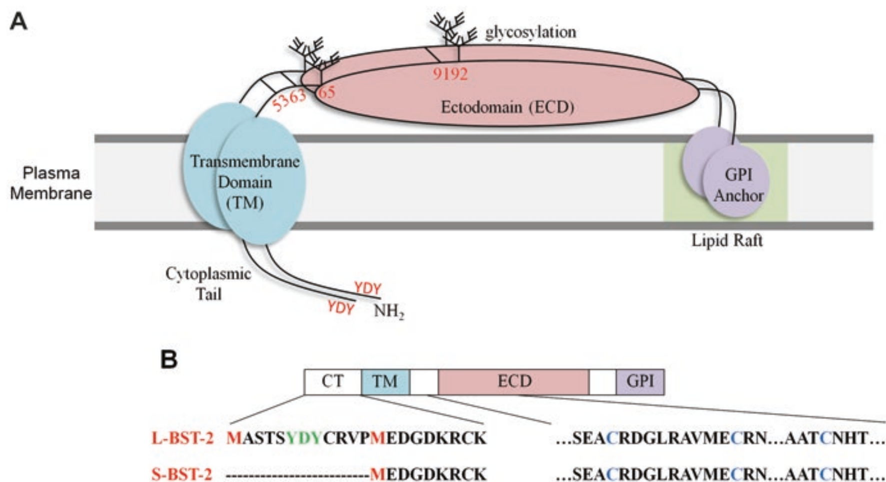

Fig. 1 *BST-2 structure and isoforms*. (**a**) BST-2 is a type II transmembrane protein with a cytoplasmic tail at the N-terminus followed by a transmembrane domain (TM), an extracellular coiled-coil ectodomain (ECD) and a glycosylphosphatidylinositol (GPI) anchor on the C-terminus embedded in lipid rafts in the plasma membrane. Numbers in red correspond to specific amino acids on the extracellular domain. Amino acids 53, 63, and 91 correspond to cysteines involved in BST-2 homodimerization through disulfide bonds. Amino acids 65 and 92 correspond to asparagines that are glycosylated, an important post-translational modification involved in BST-2 intracellular trafficking. (**b**) Box depiction of the BST-2 protein. Letters correspond to amino acids that make the cytoplasmic tail (*left*) and a section of the extracellular domain (*right*). On top is the sequence for the long isoform of BST-2 (L-BST-2) which contains the first 12 amino acids that include one translational start site (methionine) at position 1 (red letter) and a double tyrosine motif at positions 6 and 8 (6Y7X8Y, green letters). The short isoform of BST-2 (S-BST-2) lacks the first 12 amino acids and it's translated from a methionine at position 13 (red letter). Both isoforms can homodimerize because they contain cysteines involved in disulfide bonding (blue letters)

The unique topology of BST-2 allows it to retain enveloped virions on the cell membrane and to restrict viral replication (Jones et al. 2012, 2013; Casartelli et al. 2010). BST-2 consists of an N-terminal cytoplasmic tail, a transmembrane (TM) domain, and a C-terminal glycosyl-phosphatidylinositol (GPI) anchor (Kupzig et al. 2003). The TM domain and GPI anchor are separated by ~120 residues constituting the ectodomain (Fig. 1) (Hinz et al. 2010; Schubert et al. 2010). The BST-2 cytoplasmic tail contains a double tyrosine motif (6**Y**7X8**Y**) implicated in clathrin-dependent endocytosis of BST-2 (Masuyama et al. 2009) and in nuclear factor κ-B (NF-κB) activation (Galao et al. 2012; Tokarev et al. 2009; Matsuda et al. 2003; Cocka and Bates 2012). BST-2 expressed stably in healthy uninfected cell lines may not constitutively signal. However, retention of viral particles initiates phosphorylation of conserved tyrosine residues located in the cytoplasmic tail, resulting in the activation of the NF-κB signal transduction pathway (Galao et al. 2012).

The ectodomain or extracellular domain of human BST-2 contains three cysteine residues at positions 53, 63, and 91 (Hotter et al. 2013; Andrew et al. 2009, 2012) that mediate cysteine-linked dimerization (Hotter et al. 2013; Andrew et al. 2009, 2012). These cysteine residues are located at positions 58, 68, and 96 of the mouse BST-2

(Swiecki et al. 2011). Removal of the cysteine residues has been demonstrated to abolish virus tethering (Andrew et al. 2009) and to attenuate NF-κB activation, indicating that BST-2 dimerization is functionally pleotropic.

Virus tethering by BST-2 is mediated in part by the ability of BST-2 to form homodimers through covalent bonds between cysteine residues in the ectodomain of BST-2 (Santa-Marta et al. 2013). The transmembrane domain and the GPI anchor are also required for virus tethering. It is possible that BST-2 embedded in the viral membrane may dimerize with host-cell-associated BST-2 to facilitate virus tethering. However, the most likely structural arrangement that promotes efficient virion tethering is one in which the GPI anchor of cell-associated BST-2 dimer inserts into the viral membrane of budding virus (Hotter et al. 2013; Perez-Caballero et al. 2009). This arrangement allows not only for optimal virus tethering but also facilitates optimal BST-2-mediated intracellular signaling because the cytoplasmic tail from the BST-2 dimer remains inside the cell. Other possible arrangements that facilitate tethering of CHIKV particles by BST-2 include one in which the transmembrane domain of BST-2 inserts into the viral membrane (Santa-Marta et al. 2013), or one in which BST-2 buds with the virus (Habermann et al. 2010; Perez-Caballero et al. 2009; Fitzpatrick et al. 2010). Indeed, BST-2 incorporates into the membranes of nascent virions (Fitzpatrick et al. 2010).

BST-2-Mediated Retention of Nascent CHIKV Particles on Host Cells

BST-2-mediated enveloped virus tethering inhibits the release of nascent viruses to the extracellular milieu thus averting viral spread (Galao et al. 2012; Mahauad-Fernandez et al. 2014). Several studies have been described on the topic of BST-2-mediated virus tethering. However, only a handful of these studies have focused on CHIKV tethering. Both endogenously and exogenously expressed BST-2 tether nascent CHIKV particles on the surface of infected or transfected 293T cells. Because 293T cells have low BST-2 expression, this cell line is suitable for production of CHIKV, as maximum virus release is achieved with high viral titers. However, upon induction of BST-2 in 293T cells with agents such as IFNα, or ectopic expression of exogenous BST-2, virus release into the extracellular milieu is impaired. Microscopic scanning of the cell membrane is typically used to visualize virus-laden cell membrane (Fig. 2; Jones et al. 2013). Virions can be observed as tethered on the cell membrane individually or linked to each other in groups (Fig. 2b). Aside from endogenous BST-2, exogenously expressed protein is functional and tethers CHIKV virus-like particles on the cell membrane (Jones et al. 2013) or restricts CHIKV release into the culture medium (Ooi et al. 2015).

Biochemical and functional assays can also be used in the study of the effect of BST-2 on CHIKV release. BST-2-mediated effect on CHIKV particle release from host cells can be analyzed as the concentration of viral particles in the extracellular space. In this case, immunoblot analysis of CHIKV protein or reverse transcription quantitative polymerase chain reaction (RT-qPCR) are indirect assays used in determining the

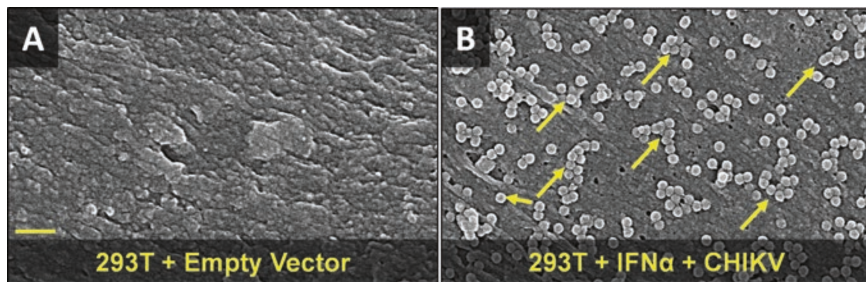

Fig. 2 *Induction of BST-2 with IFNα results in CHIKV tethering*. 293T cells were transfected with plasmids expressing (**a**) an empty vector or (**b**) treated with IFNα for 24 h followed by transfection with a CHIKV VLP (virus-like particle) plasmid. IFNα induces BST-2 which tethers CHIKV VLPs at the viral membrane. Arrows point to tethered VLPs. The scale bar represents 0.4 μm

amount of virus released from the host cell (Ooi et al. 2015). A more direct approach for assessing CHIKV particle release in the presence and absence of BST-2 is the use of nanoparticle tracking analysis (NTA), such as the NanoSight. This analytical method allows visualization and sizing of viral particles and provides information on total virus titer (Mahauad-Fernandez et al. 2014). The pitfall associated with microscopy, immunoblot, and NanoSight approaches for quantification of BST-2 tethering effect on CHIKV is that all three assays quantify total virus, including infectious and noninfectious viruses.

Assessment of BST-2 effect on the release of infectious CHIKV is accomplished using cell-based functional assays, such as endpoint dilution assay (EPDA) or quantitation of CHIKV RNA in infected cells. These assays have been used to demonstrate that expression of BST-2 in host cells significantly reduces the amount of virus released into the extracellular milieu (Mahauad-Fernandez et al. 2014).

Another alphavirus susceptible to BST-2 is the Semliki Forest virus (SFV) (Ooi et al. 2015). Unlike CHIKV that is restricted by all BST-2 isoforms (Fig. 3), the restrictive ability of BST-2 against SFV is isoform-dependent. The full length BST-2 protein efficiently retains SFV particles (Figs. 1b and 3a) whereas the short BST-2 isoform which lacks the first 12 amino acids does not inhibit SFV release (Ooi et al. 2015; Figs. 1b and 3b). It is important to note that the short isoform restricts release of other enveloped viruses such as VSV and CHIKV (Ooi et al. 2015).

How BST-2 tethers CHIKV has not been defined but two main topological arrangements could be envisioned. One arrangement consists of insertion of one end of BST-2 into the CHIKV lipid bilayer and the other end into the host cell membrane (Fig. 4a). Although BST-2 insertion into the viral and cell membranes could occur in either configuration, it appears that insertion of the BST-2 N-terminus into the host plasma membrane is the preferred orientation, as suggested by proteolytic treatment of tethered HIV virions (Perez-Caballero et al. 2009). Another possible configuration could involve insertion of both ends of the BST-2 molecules into the CHIKV lipid bilayer and dimerization of virus-borne BST-2 with host-associated BST-2 (Fig. 4b). This configuration was suggested from studies that showed the incorporation of BST-2 into HIV virions (Habermann et al. 2010; Jones et al. 2013).

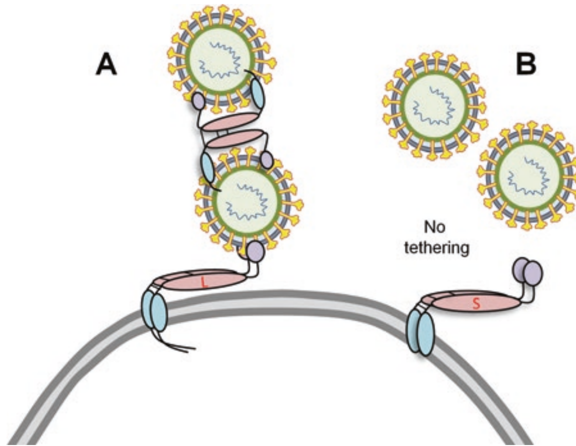

Fig. 3 *BST-2 isoform-specific restriction*. There are two different isoforms of BST-2 found in mammalian cells a (**a**) long isoform and a (**b**) short isoform that lacks the first 12 amino acids from the cytoplasmic tail. Only the long isoform of BST-2 (L) can retain SFV alphaviruses whereas the short isoform (S) is unable to tether SFV. The reason for this observation is unknown and puzzling because in infections by several retroviruses the short isoform of BST-2 presents enhanced virus tethering because of its slower turnover rate

Host-Cell-Dependent Variation in BST-2 Levels and Effect on CHIKV Release

Studies on BST-2 biology have typically been performed in HeLa and 293T cells because endogenously expressed BST-2 is elevated in HeLa cells (Mahauad-Fernandez et al. 2014; Lopez et al. 2012), whereas 293T cells have little but inducible BST-2 (Mahauad-Fernandez et al. 2014). As a consequence, HeLa cells are poor producers of BST-2-susceptible viruses compared to 293T cells (Mahauad-Fernandez et al. 2014; Ooi et al. 2015; Kaletsky et al. 2009; Neil et al. 2007). Indeed, release of total CHIKV particles (Ooi et al. 2015) and infectious CHIKV (Mahauad-Fernandez et al. 2014) is significantly hampered in HeLa cells that express a high level of endogenous BST-2. Analyses of different cell types reveal that BST-2 has a broad-spectrum inhibitory effect on CHIKV release from various human (HeLa, 293T, and U937) and murine (MEF and macrophages) cell types (Mahauad-Fernandez et al. 2014). Regardless of the cell type, adherent or suspension, endogenous BST-2 restricts release of infectious CHIKV particles and induction of BST-2 with IFNα potentiates restriction of CHIKV particle release (Mahauad-Fernandez et al. 2014). These findings indicate that BST-2 could potentially prevent CHIKV particle release and spread in most cell types, albeit with some variations in efficiency. Indeed, studies from different laboratories using human epithelial cells, including HeLa and 293T cells have been used to demonstrate the link between the levels of endogenous BST-2 in various cells to the amount of virus released from such cells. It is plausible that the level of BST-2 in HeLa, 293T, and U937 cells may contribute to their varied abilities to respond to CHIKV infection (Mahauad-Fernandez et al. 2014).

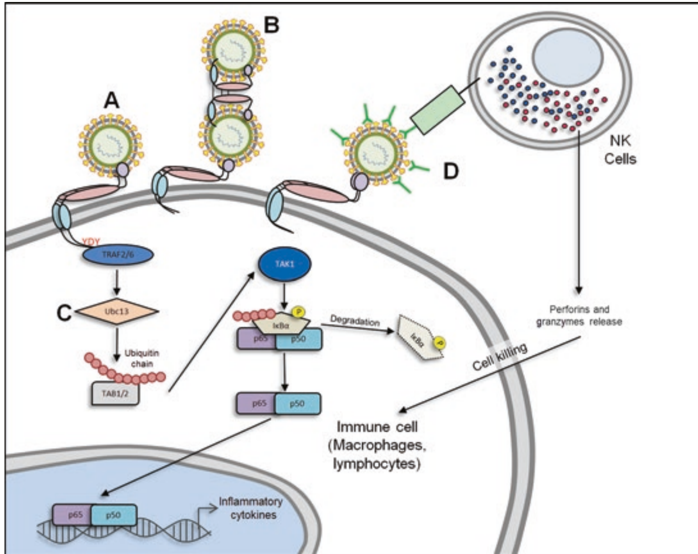

Fig. 4 *BST-2-mediated CHIKV tethering and antiviral immune responses*. BST-2 averts enveloped virus release by tethering viruses to the plasma membrane of infected cells. There are two possible topologies utilized by BST-2 to tether viruses. (**a**) One consists in the insertion of the GPI anchor into the viral double lipid membrane while the transmembrane domain remains in the cell membrane. (**b**) The second conformation consists in the incorporation of BST-2 proteins into the viral membrane. Virus-associated BST-2 can homodimerize to cell-associated BST-2 leading to virus tethering. (**c**) A topology in which the transmembrane domain is inserted into the viral membrane is not likely because the cytoplasmic tail is necessary for BST-2 induction of NF-κB. Virus tethering by BST-2 induces the activation of NF-κB through the YxY motif that interacts with TRAF1/2 and requires Ubc13 to generate a ubiquitin chain used as a scaffold by Tab1/2 to recruit TAK1 which phosphorylates IkBα. Phosphorylated IkBα is then ubiquitinated and degraded liberating p65/p50 NF-κB which translocates to the nucleus to transcribe inflammatory cytokines. (**d**) Furthermore, CHIKV tethering by BST-2 may induce ADCC by increasing the amount of envelope proteins (yellow spikes on virus) available to be recognized by anti-Env antibodies (green Y) which upon binding to their specific receptor leads to the activation and degranulation of effector cells such as natural killer (NK) cells that release perforins and granzymes to lyse and kill infected cells

BST-2-Mediated Inhibition of CHIKV Replication: An Arms Race

In addition to its role in preventing nascent CHIKV particle release, BST-2 has an additional function in CHIKV pathogenesis. Animal model studies showed that deletion of the BST-2 gene in mice (BST-$2^{-/-}$) results in increased susceptibility of mice to infection with the vaccine strain of CHIKV (CHIKV 181/25) (Mahauad-Fernandez et al. 2014). Compared to BST-2-expressing wild-type (WT) mice, BST-$2^{-/-}$ mice have a significantly higher viral load at the site of virus inoculation (Mahauad-Fernandez et al. 2014). Viral load in this case was quantified as viral RNA or infectious particles (Mahauad-Fernandez et al. 2014). In line with high

inoculation site viral load, systemic spread of CHIKV to most distal organs, except the heart was enhanced in BST-2$^{-/-}$ mice (Mahauad-Fernandez et al. 2014). In the heart, expression of BST-2 results in high CHIKV replication and high viral load (Mahauad-Fernandez et al. 2014). These findings demonstrate that BST-2 plays an important role in determining the pathogenicity of CHIKV in vivo, although in a tissue-specific manner. The altered replication characteristics of CHIKV in vivo do not affect the overall significant increases in viral load in BST-2$^{-/-}$ mice. The failure of CHIKV to spread to the heart despite high plasma viremia suggests that BST-2 functions in a tissue-/cell-type–specific manner.

How BST-2 protects the host from CHIKV infection is yet to be determined. However, the mechanism may combine specific host–virus interactions along with other intrinsic abilities of each cell type to respond to CHIKV infection, after all, the antiviral functions of BST-2 are cell-/tissue-type dependent (Mahauad-Fernandez et al. 2014; Erikson et al. 2011). As such, cells with high levels of interferon alpha (IFNα) are less susceptible to CHIKV and mice deficient in IFNα receptor are highly susceptible to CHIKV infection. Although the IFN system plays a significant role in controlling CHIKV, the cross-talk between BST-2 and IFN is complex and may contribute to CHIKV pathogenesis. Following acute CHIKV infection, levels of BST-2, IFNα, and IFNγ are correlatively increased in WT mice. However, in BST-2$^{-/-}$ mice, IFNα and IFNγ expression are significantly reduced (Mahauad-Fernandez et al. 2014). IFNs are critical for the control of CHIKV infection (Briolant et al. 2004; Schilte et al. 2010; Couderc et al. 2008; Gardner et al. 2012; Partidos et al. 2011; Wauquier et al. 2011), therefore it is likely that the anti-CHIKV activity of IFN may require BST-2 (Mahauad-Fernandez et al. 2014). Also in discord between WT and BST-2$^{-/-}$ mice is the level of CD40 ligand, which increases in WT but is reduced in BST-2$^{-/-}$ mice following acute CHIKV infection. These findings suggest a paradigm where BST-2 induces and/or amplifies innate immune responses against CHIKV (Fig. 4c; Galao et al. 2012; Tokarev et al. 2013).

It is not surprising that BST-2 may orchestrate immune signaling in infected mice because it is known that BST-2 through its double tyrosine motif (6Y7X8Y) activates the promiscuous transcription factor NF-κB (Galao et al. 2012; Tokarev et al. 2013). BST-2-mediated activation of NF-κB leads to the transcription and production of a plethora of NF-κB-dependent genes or molecules. Interestingly, the promoter of CD40 ligand contains NF-κB binding sites (Srahna et al. 2001; Schubert et al. 2002). It is therefore possible that regulation of CD40 ligand synthesis and function may depend on the BST-2-NF-κB signaling pathway (Fig. 4c). The observed BST-2-mediated regulation of CD40 ligand expression is significant given that CD154/CD40L-mediated activation of B cells by T cells via immunoglobulin class switching from IgM to IgG and IgA is critical for immunity against viruses. Therefore, in the absence of BST-2, CHIKV could evade T-cell dependent IgG and IgA responses by downregulating CD40 ligand synthesis, as has been put forth for other viral infections (Lopez et al. 2012; Kaletsky et al. 2009; Neil et al. 2007).

Aside from eliciting an immune signaling cascade, BST-2 has been shown to provoke antibody-dependent cellular cytotoxicity (ADCC) in retrovirus infected cells. BST-2-mediated ADCC is postulated as a potential mechanism of viral clearance

(Pham et al. 2014; Arias et al. 2014; Alvarez et al. 2014). Whether this phenomenon occurs in CHIKV-infected cells is yet to be determined. Nonetheless, it is possible that the Env protein of CHIKV may contain epitopes that could be recognized by cytotoxic-inducing antibodies. Binding of these antibodies to viral Env could elicit degranulation of effector cells and destruction of infected cells, as demonstrated (Fig. 4d) for murine retroviruses (Alvarez et al. 2014; Liu et al. 1995). Although it is proposed that BST-2-mediated ADCC may elicit viral clearance, it is possible that ADCC-mediated lysis of virus-infected cells may have pleotropic effects. Released viral particles may indeed result in enhanced infection through dissemination to distal sites.

It is worth mentioning that the short isoform of BST-2 which lacks a YxY motif involved in BST-2 endocytosis (Fujita et al. 2012; Rollason et al. 2007) is more resistant to viral antagonists compared to the long isoform (Cocka and Bates 2012; Weinelt and Neil 2014). Because the YxY motif plays a role in the induction of inflammatory cytokines through NF-κB (Galao et al. 2012; Tokarev et al. 2013; Fig. 4c), it is possible that the short BST-2 isoform may not induce cytokines and cytotoxic processes such as ADCC (Fig. 4d) or complement-dependent cytotoxicity (CDC), hence resulting in viral dissemination as has been shown in vivo with the Friend retrovirus (Li et al. 2014).

Although BST-2 inhibits replication of CHIKV 181/25, it is unknown whether wild-type CHIKV is susceptible to the antiviral effect of BST-2. Studies using wild-type CHIKV are needed for complete understanding of the role of BST-2 in CHIKV pathogenesis. Additional investigation is warranted to determine the mechanisms of BST-2-mediated restriction of CHIKV replication. It is also necessary to determine if other potent restriction factors such as TRIM-5α, APOBEC3 family of cytidine deaminases, and SAMHD1 that inhibit infection by other viruses play any role in CHIKV pathogenesis.

CHIKV Antagonism of BST-2

Despite BST-2 potent activity against different viral families, viruses have devised various means to neutralize BST-2 effects. Whereas BST-2 tethers CHIKV on the membrane of infected cells, expression of CHIKV nonstructural protein 1 (nsP1) neutralizes BST-2-mediated CHIKV tethering (Jones et al. 2013). The CHIKV nsP1 is a multifunctional protein involved in viral mRNA capping and synthesis of CHIKV minus-strand RNA genomes (Salonen et al. 2003). The CHIKV envelope (E) protein E1 and nsP1 colocalize with BST-2 at the cell membrane and in intracellular compartments (Fig. 5a, i) whereas other CHIKV proteins such as nsP2, nsP3, nsP4, the capsid, E3, E2, and 6K proteins do not colocalize with BST-2 (Jones et al. 2013) (Fig. 5b–h). In spite of the colocalization between E1 and BST-2 and nsP1 and BST-2, only nsP1 antagonizes the tethering function of BST-2, thus enhancing CHIKV release (Jones et al. 2013).

The mechanism used by nsP1 to neutralize the actions of BST-2 is unclear (Fig. 6). However, nsP1 is known to decrease BST-2 expression at the cell surface (Jones et al. 2013; Fig. 6a), similar to the action of HIV Vpu on BST-2 (Dube et al. 2010a, b).

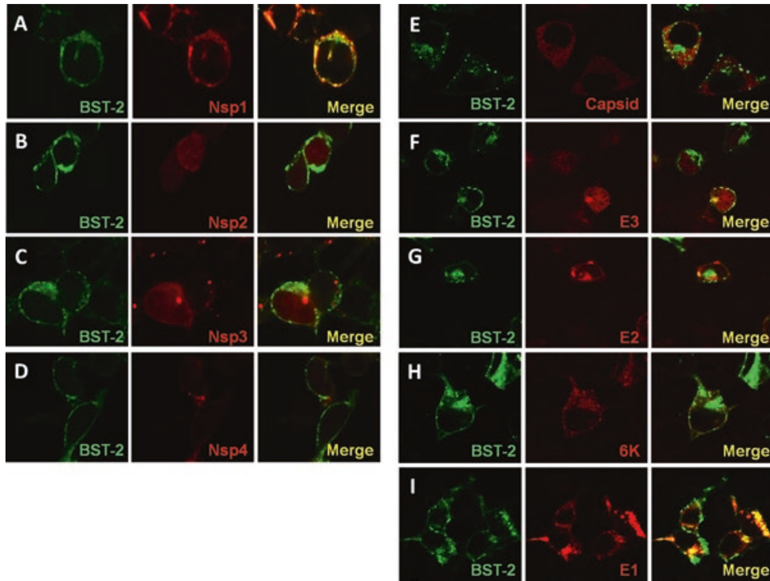

Fig. 5 *Colocalization of CHIKV proteins with BST-2*. CHIKV encodes for nonstructural proteins (nsP), envelope proteins (E), a capsid protein, and 6K protein. (**a**–**d**) With respect to nonstructural proteins, BST-2 only colocalizes with nsP1 mainly at the cell membrane but also in intracellular compartments (**a**); on the other hand, BST-2 does not colocalize with nsP2 (**b**), nsP3 (**c**), or nsP4 (**d**). (**e**–**i**) With respect to structural proteins, BST-2 does not colocalize with capsid proteins (**e**), E3 (**f**), E2 (**g**), and 6K (**h**); but BST-2 does colocalize with E1. Images were taken at 63×

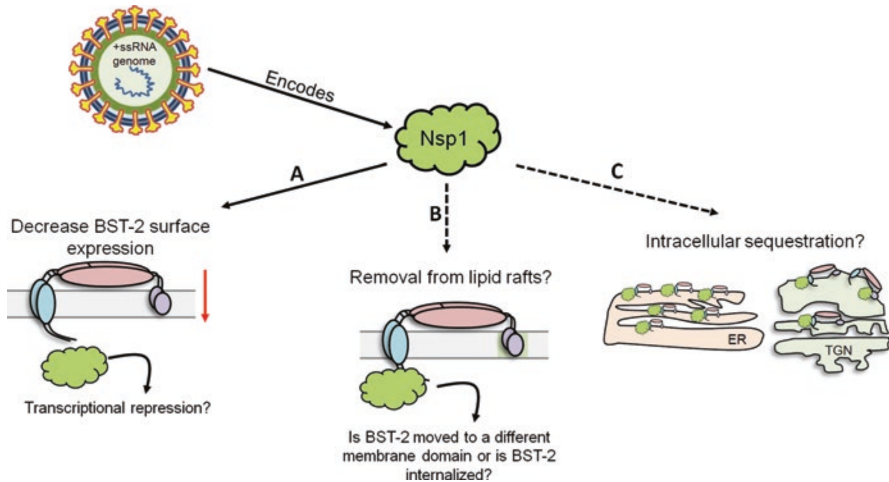

Fig. 6 *Proposed model for CHIKV nsP1-mediated neutralization of BST-2*. CHIKV encodes for a nonstructural protein 1 (nsP1) which is involved in the synthesis of the negative-strand RNA genomes as well as capping the positive-strand RNA genome. (**a**) With respect to CHIKV anti-BST-2 functions, nsP1 was found to decrease the expression of BST-2 at the plasma membrane though the exact mechanism is not known. Perhaps nsP1 leads to the transcriptional repression of BST-2. (**b**) CHIKV nsP1 may also antagonize BST-2 by removing BST-2 from lipid rafts allowing CHIKV release. CHIKV nsP1 may either internalize BST-2 or move BST-2 to a different membrane domain that is not a site of CHIIKV budding. (**c**) Moreover, nsP1 may retain BST-2 in intracellular compartments preventing BST-2 normal trafficking to the cell membrane as has been reported for HIV Vpu protein. Dashed arrows correspond to hypothetical models of nsP1 antagonism of BST-2

Furthermore, treatment of cells with IFNα, which induces BST-2 expression, potentiates CHIKV tethering at the cell membrane. CHIKV infection is known to diminish IFNα production (Couderc et al. 2008) suggesting that this may be an attempt by the virus (1) to indirectly reduce BST-2 expression and promote its release and (2) to prevent BST-2 incorporation into CHKV virions as has been suggested with HIV virions (Fitzpatrick et al. 2010). In addition, nsP1 preferentially binds to lipids (Ahola et al. 1999; Lampio et al. 2000); it is therefore tempting to speculate that nsP1 may function to remove BST-2 from lipid rafts (Fig. 6b). It is also plausible that nsP1 may sequester BST-2 intracellularly inasmuch as BST-2 and CHIKV colocalize to perinuclear compartments such as the ER and TGN (Jones et al. 2013; Fig. 6c). These viral antagonistic actions have been previously reported for HIV Vpu (Rollason et al. 2013; Arias et al. 2012; Dube et al. 2011; Janvier et al. 2011).

Conclusion

In order to control CHIKV infection and CHIKV-associated diseases, we are in need of discovery and design of efficacious and affordable anti-CHIKV therapy or vaccines. As detailed in this chapter, the host virus restriction factor—BST-2—is intrinsic to the host and acts without priming to limit infection by CHIKV and to prevent release of nascent CHIKV particles to the extracellular milieu. Because BST-2 elicits activation of signal transduction cascade driven by the master transcription factor NF-κB, it is possible that BST-2 constitute a network of communication that may act in a tissue-specific context or in association with many other host molecules as exemplified by the divergent role of BST-2 in infection of heart tissues. Currently, many questions remain about the mechanisms of BST-2-mediated (1) inhibition of CHIKV replication and release, (2) modulation of CHIKV-induced inflammatory response and immune surveillance, (3) precise molecular mechanisms of BST-2 neutralization by CHIKV, and (4) the ability of BST-2 to restrict wild-type CHIKV. Answers to these questions will fundamentally push the frontiers of our understanding of the role of restriction factors in CHIKV pathogenesis.

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