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Chikungunya virus nonstructural protein 1 as an antiviral target

Kovacikova, K.

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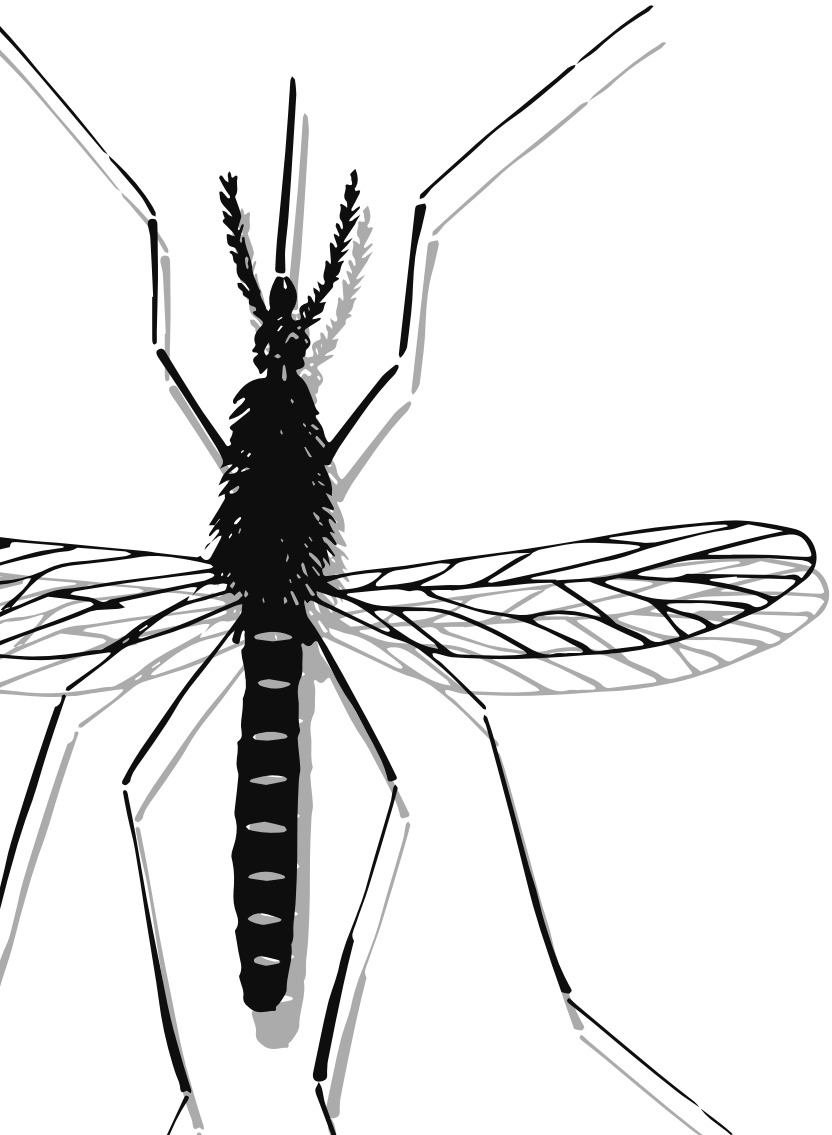

CHAPTER 1

General Introduction

Alphaviruses as human pathogens

The vast majority of (re)emerging viral infectious diseases are caused by viruses with an RNA genome. Among these, arthropod-borne (arbo)viruses are especially important, since they can result in high morbidity and mortality of people and livestock. They alternate between vertebrate and invertebrate hosts which makes their replication cycles unique and fundamentally different from those of single-host RNA viruses. Mosquito-borne alphaviruses are divided into arthritogenic and encephalitic alphaviruses. The arthritogenic alphaviruses include chikungunya virus (CHIKV), Semliki Forest virus (SFV), Sindbis-like viruses (SINV), Mayaro virus (MAYV), o'nyong-nyong virus (ONNV), Barmah Forest virus (BFV) and Ross River virus (RRV), among others, and are a major cause of infectious arthritis-like disease worldwide. They are typically endemic in Africa, South and Southeast Asia, parts of Central and South America as well as southern and northern Europe. The encephalitic alphaviruses include Venezuelan equine encephalitis virus (VEEV), Eastern equine encephalitis virus (EEEV) and Western equine encephalitis virus (WEEV). These viruses circulate in Central, South and North America. While relatively rare in humans, they can cause one of the most severe acute viral infections with neurological symptoms. These viruses can also be spread by aerosols, and therefore their potential misuse for biological warfare is of great concern, which is why they are currently placed on the US Select Agents and Toxins list.

The first symptom of an infection with arthritogenic alphaviruses is a febrile illness, which can progress to rheumatic disease, described as polyarthralgia and/or polyarthritis and can be chronic and debilitating. CHIKV was originally isolated in 1952/1953 from the serum of a febrile patient in the present-day Tanzania (1). It is likely that CHIKV epidemics occurred before this period but were misdocumented as dengue fever outbreaks (2). CHIKV is genetically closely related to ONNV, which has so far been geographically restricted to the African continent. CHIKV and ONNV infections cause similar symptoms, which is reflected in the native African names for CHIKV and ONNV that mean "that which bends up" and "weakening of the joints", respectively.

CHIKV persists in sylvatic or enzootic cycles in sub-Saharan Africa, where the transmission occurs between multiple forest-dwelling *Aedes* spp. mosquitoes and non-human primates (NHPs). In sylvatic cycles, humans are considered incidental hosts and are sporadically infected by a spillover from sylvatic vectors. Initially, phylogenetic

studies identified two enzootic CHIKV lineages: West African and East/Central/South African (ECSA) (3). An Asian lineage, likely to have originated from the ECSA lineage, was first isolated in 1958 and implicated in outbreaks in India and Southeast Asia during the 1960s (4). In both Africa and Asia, CHIKV epidemics occurred with intervals of 7 to 20 years between the 1950s and 2000s. The Indian Ocean lineage (IOL), a descendant of the ECSA lineage, was responsible for the explosive CHIKV outbreak in coastal Kenya in 2004, from where it spread over several Indian Ocean islands and subsequently to large parts of South and South East Asia. Since then, it has caused recurrent and sometimes large epidemics, especially in the Indian subcontinent and in Southeast Asia (5, 6).

Like for other arboviruses such as ONNV, Zika virus (ZIKV) and dengue virus (DENV), humans are not considered dead-end hosts and the human-mosquito-human transmission cycle is responsible for sustaining high CHIKV transmission rates in the human population (7). Prior to 2005, *Ae. aegypti* was recognized as the only vector for CHIKV transmission in urban areas. The amino acid substitution A226V in the E1 glycoprotein (E1-A226V), which was first detected during the La Reunion epidemic in 2005-2006, led to a resurgence of the outbreak on this island and facilitated virus transmission by *Ae. albopictus* (8). After 2006, CHIKV has expanded into the temperate regions of the world via infected air travellers and in part due to its enhanced ability to infect *Ae. albopictus* mosquitoes, which – for example- resulted in recent European outbreaks in Italy in 2007 and 2017 (9, 10) and in France in 2010, 2014 and 2017 (11-13) (Fig. 1). In late 2013, on the Island of St. Martin in the Caribbean, autochthonous CHIKV transmission was reported for the first time in the Americas (14). The outbreak was caused by a CHIKV strain belonging to the Asian lineage that had been circulating in the urban cycle at least since the 1950s (15). Interestingly, Brazil has experienced two independent introductions of CHIKV strains. Since 2014, strains belonging to the Asian lineage and to the ECSA genotype have been co-circulating in the Brazilian territory (16).

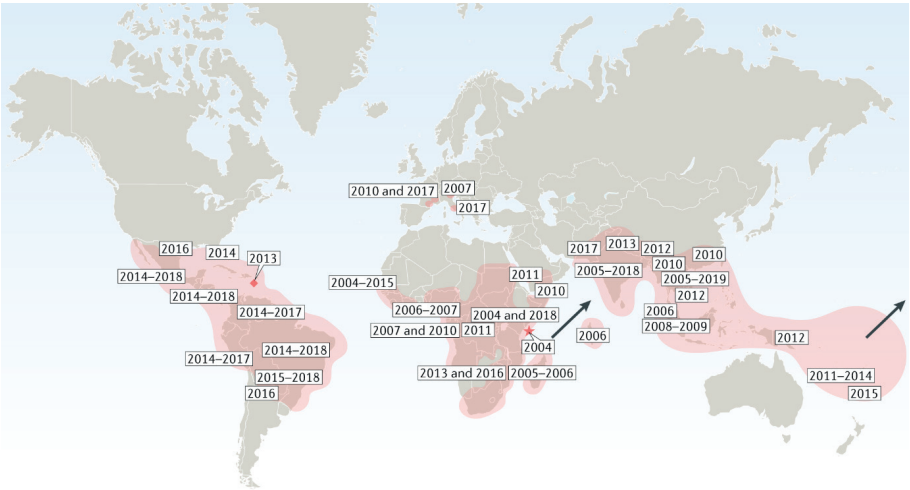

Figure 1: Emergence and spread of the 2004-2020 CHIKV epidemic. In 2004, CHIKV re-emerged on Lamu Island, in Kenya (red star). The epidemic expanded in Africa and spread eastwards across Indian Ocean islands and in Asia. CHIKV reached the Americas in late 2013, when the first autochthonous transmission was reported on the St. Martin Island in the Caribbean (red diamond). From there, CHIKV spread into Central and South America. Throughout the CHIKV epidemic, small regional outbreaks have also occurred in Italy and France and southern USA. Reused with permission from (17).

Disease manifestations, clinical outcome and economic impact of CHIKV infection

CHIKV typically causes a self-limiting febrile illness known as chikungunya fever (CHIKF) associated with potentially chronic debilitating joint and muscle pain. CHIKV outbreaks are characterized by high attack rates, with up to 30 – 75% population being affected with CHIKV at a time (18). Most CHIKV infections are symptomatic and the number of people who develop symptoms is on average higher than for those infected with flaviviruses, for example. The incubation period for CHIKV infection ranges from 2 to 7 days. The onset of fever is often very abrupt and the fever typically lasts up to 7 days. In some cases, the clinical symptoms can resemble those of diseases caused by other arthropod-borne agents, such as DENV. Polyarthralgia usually develops around the same time and primarily affects the peripheral joints such as fingers, toes, wrists, elbows and knees. Some individuals also develop an itchy maculopapular skin rash, affecting mainly the torso and the extremities (19). The disease is associated with high morbidity, but is rarely fatal. In general, patients with chronic CHIKV recover

within 3 – 24 months (20). However, the La Reunion epidemic brought to light atypical manifestations such as severe neurological complications, including encephalitis and Guillain-Barre syndrome (21). Such forms of CHIKF are less frequent and limited to subgroups of patients like the elderly, the very young and people with underlying comorbidities (22, 23). Infections acquired via mother-to-child transmission, first reported during the La Reunion outbreak, can be fatal or involve severe neurological sequelae in foetuses and young infants (24). The economic impact of CHIKV is high, especially given the burden of disease and related consequences for work and school absenteeism. There is an immediate cost to the patient due to lost productivity and associated treatment costs.

CHIKV genome structure and replication cycle

CHIKV is a small, enveloped (+) strand RNA virus that belongs to the Old Word (Semliki Forest virus-like) group of arthritogenic alphaviruses within the *Togaviridae* family. The CHIKV genome comprises a single-stranded RNA genome of 11,800 nucleotides, with a 5' 7-methylguanosine cap and a 3' poly-A-tail. The genome contains two open reading frames (ORFs) that are flanked by 5' and 3' untranslated regions (UTRs) (25). The 5' UTR comprises 76 nucleotides while the 3' UTR contains as many as 526 nucleotides. In addition, a third UTR of 68 nucleotides is found between the two ORFs and carries a promoter sequence to direct the transcription of the 26S subgenomic RNA (26). The first ORF, constituting approximately the 5' two-thirds of the genome, encodes for nonstructural proteins 1-4 (nsPs) that are part of the viral replicase complex. The second ORF, which is expressed from the 26S mRNA, covers the 3' one-third of the genome and encodes for six proteins: capsid (C), E1, E2, E3, 6K and transframe (TF) protein (27), the latter being a product of-1 ribosomal frameshifting in the sequence encoding the 6K protein (Table 1). The CHIKV virion is formed by a glycoprotein shell enclosing a host-derived lipid bilayer and an icosahedral nucleocapsid composed of 240 units of C proteins. The CHIKV envelope carries 80 sets of spikes, each made up of three E1-E2 heterodimers (28).

Table 1: CHIKV proteins and their functions

Protein	Function	Reference
nsP1	guanine-N7-methyltransferase	(42)
	guanylyltransferase	(43)
	membrane association	(44)
nsP2	nucleoside triphosphatase	(45)
	RNA 5' triphosphatase	(46)
	helicase	(47)
	protease	(48)
	innate immune evasion/host shut-off	(49, 50)
nsP3	ADP-ribosylhydrolase	(51, 52)
	replication	(53)
nsP4	RNA-dependent RNA polymerase	(54, 55)
	terminal adenylyltransferase	(56)
C	nucleocapsid formation	(37)
E1	membrane fusion	(57, 58)
E2	receptor binding	(59)
E3	particle assembly	(60)
6K	ion channel	(61, 62)
TF	(particle assembly)	(39)

CHIKV enters cells via E2-mediated attachment to a host cell receptor (Fig. 2). The matrix remodelling-associated protein 8 (Mxra8) has been identified as a receptor for multiple arthritogenic alphaviruses (29). Glycosaminoglycans (GAGs), which are expressed on many susceptible cell types, also serve as an attachment factor and aid CHIKV entry into the cells. Following viral attachment to the cells, viral particles are internalized mainly by clathrin-mediated endocytosis. Endosomal acidification triggers conformational changes in the viral glycoproteins which lead to the insertion of the E1 fusion loop into the host membrane (30). The membrane fusion process occurs rapidly and is highly dependent on the presence of cholesterol (31). Following release into the cytoplasm, the incoming viral RNA (vRNA) gets directly translated to produce the P1234 replicase polyprotein precursor. Nevertheless, only about 10% of the translation events produce the full P1234 polyprotein precursor, whereas the rest yields a P123 polyprotein (25). To produce P1234, translational readthrough has to occur due to the presence of an opal (UGA) stop codon between the nsP3- and nsP4-coding sequences, which is thought to regulate the levels of nsP4 (32). The P1234 polyprotein precursor is cleaved in *cis* into P123 and nsP4 by a protease domain in

nsP2. P123 together with nsP4 forms an 'early replication complex', which is short-lived, synthesizes a full-length (-) strand vRNA (33) and induces the formation of virus replication compartments termed spherules. Spherules serve as the sites for vRNA replication and are initially targeted to the plasma membrane by the nsP1 portion of P123, while later during infection they form endosome-like compartments in the cytosol (34), also called virus-induced type 1 cytophathic vacuoles (CPV-1) (35, 36). Later in the replication cycle, P123 gets further cleaved in *trans* into the individual nsPs, which can assemble into a nsP1-4 'late replication complex'. The nsPs can be

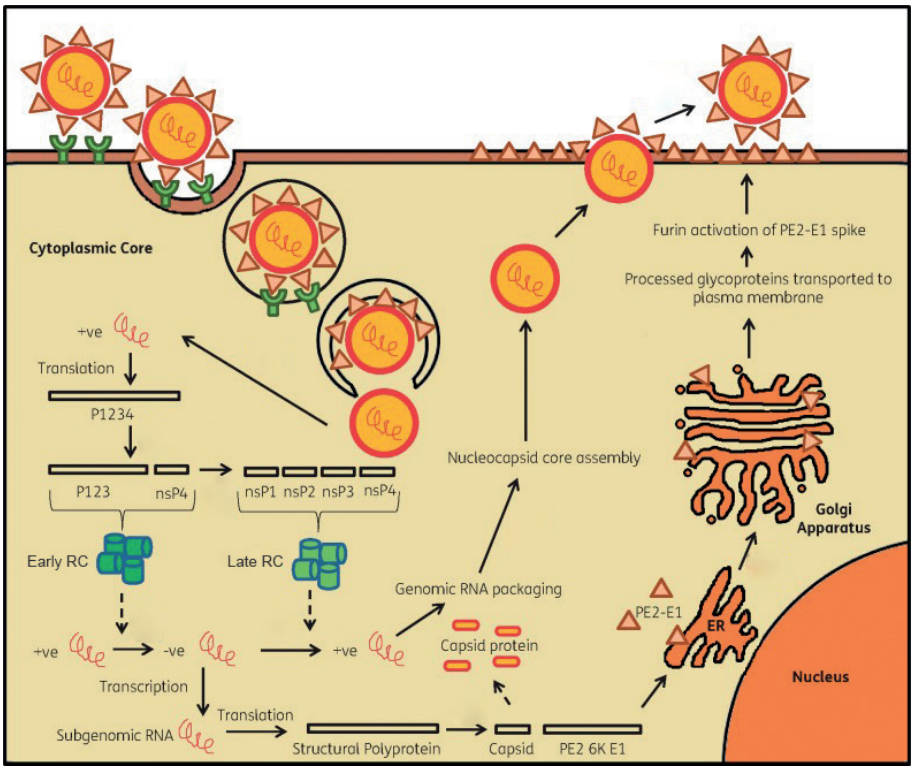

Figure 2: CHIKV replication cycle. In short, CHIKV enters cells mainly via clathrin-mediated endocytosis upon which the nucleocapsid is released into the cytosol. The incoming vRNA is translated to produce P1234 polyprotein precursor, which is first cleaved into P123 and nsP4 and forms an early RC, and then fully processed nsP1-4 form a late RC. The early RC is responsible for the synthesis of (-) strand vRNA and spherule formation while the late RC carries out the synthesis of genomic (+) strand vRNA and 26S subgenomic (+) strand vRNA. Following translation of the structural polyprotein derived from the 26S subgenomic (+) strand vRNA, the C protein is autocatalytically cleaved and forms a nucleocapsid upon interaction with newly synthesized genomic (+) strand vRNA. The E1 and E2 glycoproteins undergo a series of post-translational modifications in the Golgi secretory pathway, following which they assemble at the host plasma membrane. Finally, the CHIKV nucleocapsid acquires a host-derived lipid bilayer with E1 and E2 glycoproteins upon release from the host cell. Adapted from (63).

found at the neck of spherules, which harness dsRNA intermediates in order to protect them from detection by the cytoplasmic innate immune sensors. The late replication complex is responsible for the synthesis of genomic (+) strand vRNA and 26S subgenomic (+) strand vRNA. The 26S subgenomic (+) strand vRNA encodes the structural polyprotein C-E3-E2-6K(TF)-E1. Following autocatalytic cleavage of the C protein from the structural polyprotein (37), the C protein in the cytosol interacts with newly synthesized genomic (+) strand vRNA to initiate the formation of nucleocapsids (38). Subsequent synthesis of the structural polyprotein generates a majority product E3-E2-6K-E1 and a minority product E3-E2-TF due to ribosomal frameshifting (27, 39). A signal sequence in the N-terminal region of E3 directs the major and minor structural polyproteins to host secretory pathways where they get modified. Cleavage by host proteases results in pE2 (E3-E2); 6K or TF; and E1 (40). During the transit through the Golgi secretory pathway, E1 and E3-E2 undergo conformational changes and post-translational modifications including palmitoylation, N-linked glycosylation and E3 release due to host furin cleavage. At the late phase of infection, type 2 cytophathic vacuoles (CPV-2) transport E1 and E2 glycoproteins from the *trans*-Golgi network to the plasma membrane (41). Finally, fully formed nucleocapsids carrying full-length (+) strand genomic RNA assemble at the plasma membrane with mature E1 and E2 glycoproteins to form a mature virion, which is released from the host cell (33).

Immunopathology of CHIKV infection during the acute phase

Upon a bite of an infected mosquito, CHIKV primarily replicates in dermal fibroblasts. From the skin, the virus migrates to other parts of the body via the lymphatic system until it reaches the bloodstream. Through the bloodstream, CHIKV reaches joint, muscle and bone tissue, which represent the main sites linked to the most commonly observed clinical manifestations (Fig. 3). While the clinical course of CHIKV infection is rather well-documented, molecular details on how precisely CHIKV causes disease are not well understood. Many of the antiviral responses that are involved in immunopathology are also essential for the clearance of viral infection, which has important implications for the development of therapeutics.

During acute chikungunya fever, patients experience peak viremia at day 2 after the initiation of symptoms. The viremia declines sharply during days 3 and 4, and becomes undetectable by day 5 (64). CHIKV reaches a high viral load during the acute

phase of infection (> 10^6 to 10^{10} viral particles per millilitre of blood), with particularly high titres in the elderly. The strong decline in viremia before the generation of potent neutralizing antibodies is consistent with an important role for the early interferon (IFN) response. The recapitulation of CHIKV infection in a mouse model with abrogated IFNα/β signalling resulted in severe CHIKV infection (65). Type I IFNs, such as IFNα or IFNβ, are highly efficient in limiting CHIKV replication. To counteract the host IFN response, alphaviruses have evolved suppression and evasion mechanisms, for example by eliciting a nsP2-mediated host transcriptional shutoff in infected cells (66, 67). Interestingly, the anti-alphaviral activity of type I IFNs is less effective at lower temperatures. The peripheral disease of limbs and extremities appears to be a consequence of enhanced CHIKV replication in these sites, owing to their slightly lower body temperature (68).

Experimental evidence suggests that fibroblasts could be the principal source of type I IFNs during CHIKV infection (69). Skeletal muscle, joint and dermal fibroblasts are the primary sites of CHIKV replication early in infection (70). The presence of Mxra8 on a vast array of different cell types (71) indicates that monocytes, macrophages, endothelial cells or nerve cells can also serve as a potential reservoir. The acute phase of CHKV infection in humans results in increased pro-inflammatory cytokines including interleukin 6 (IL-6), monocyte chemotactic protein 1 (MCP-1), IFNα, IFNγ, IFNγ-induced protein 10 (IP-10) and monokine induced by IFNγ (MIG) (72, 73). Some of these molecules serve as chemokines and are responsible for recruiting leukocytes to the sites of infection and triggering cellular responses.

High plasma levels of IFNγ, IL-4, IL-7 and IL-12p40 are associated with the onset of adaptive immunity. The primary response to acute CHIKV infection is also characterized by a strong up-regulation of $CD8^+$ cytotoxic T cells (74) and the development of neutralizing antibodies (Abs). Neutralizing anti-CHIKV IgM Abs develop as early as 4 days after the onset of symptoms and play the predominant neutralizing role in the first phase of infection (75). Neutralizing anti-CHIKV IgG Abs, consisting primarily of the IgG3 isotype, are usually detected when the virus is cleared (7-10 days after the onset of symptoms) and persist for many years. A robust IgG3 response is linked to a more severe acute phase but reduces the likelihood of developing persistent arthralgia (76). These observations indicate that the adaptive immune response plays an important role in controlling CHIKV-induced arthritis, further suggesting a direct link between viral infection and joint inflammation (77) (Fig. 3). The acute symptoms usually resolve within 2 weeks, but incapacitating arthralgia may linger for weeks to months and in some cases even years.

Mechanisms of CHIKV persistence and tissue inflammation during a chronic phase

Chronic chikungunya arthritis (CCA) is often described as a rheumatic syndrome as the pathogenesis of CCA and rheumatoid arthritis may involve similar mechanisms. Long-lasting arthralgia, in particular, is a clinical sign that distinguishes CHIKV from DENV infection. Up to 40% of infected patients ultimately develop CCA. The increased probability of developing CCA depends on predisposing factors such as comorbidities (e.g. diabetes), older age (>35-45 years for joint pain), and high viremia and severe disease during the acute phase (78).

CHIKV infection is cytopathic and results in direct tissue injury. Alphavirus-associated arthritides are predominantly caused by mononuclear cells such as monocytes and macrophages, with some contribution of B cells, T cells and natural killer (NK) cells (17). Studies in a macaque model indicate that macrophages are the likely main cellular reservoir of persistent CHIKV. At later stages of infection, CHIKV was found to persist in joints, muscles, lymphoid organs and liver for at least 3 months after viral inoculation (79). In addition, musculoskeletal cells and fibroblasts have also been shown to harbour persistent viral RNA (80). CHIKV persistence in these target sites is likely responsible for the long-lasting disease symptoms. There are several ways in which pathogenic CHIKV can establish persistence, such as by evasion of neutralizing antibodies produced by B cells (81) or by evasion of antiviral $CD8^+$ T cell immunity (82). CHIKV-apoptotic blebs, among others, have also been implicated in virus persistence by allowing the virus to escape the host immune response and infect neighbouring cells (83). CHIKV-induced arthropathies likely emerge from innate and adaptive immune responses stimulated by the virus and/or viral material (RNA or protein) in joint tissues (84). Strikingly, researchers have struggled to isolate infectious virus from patients with chronic disease. One study reported the isolation of CHIKV RNA and protein from synovial macrophages of 1 patient 18 months after infection (85), illustrating the difficulty to obtain infectious virus from chronic patients.

CCA has been associated with increased circulating levels of pro-inflammatory cytokines including IL-6, granulocyte macrophage colony-stimulating factor (GM-CSF), IFNα and IL-17 (86). In CCA, plasma levels of IL-6 and GM-CSF have been found to be significantly higher in patients with persistent arthralgia compared to those who recovered (87). CHIKV-infected monocytes in the later stage of infection are the major source of pro-inflammatory mediators IFNα, IL-12, CC-chemokine ligand 2 (CCL2)

and CXC-chemokine ligand 10 (CXCL10) (88), while macrophages produce mostly IL-6 and tumour necrosis factor (TNF) (89). A major challenge for the field has been the identification of proinflammatory immune mediators that could be targeted with therapeutics without compromising their antiviral functions.

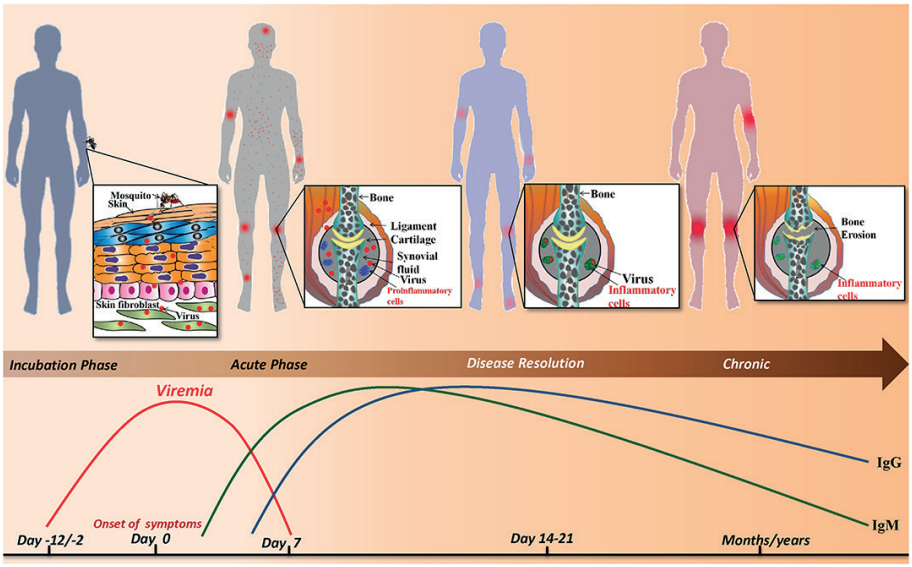

Figure 3: The clinical course of CHIKV infection. Following a bite of an infected mosquito, CHIKV primarily replicates in dermal fibroblasts from where it spreads to the rest of the body via the lymphatic system and the bloodstream. CHIKV mostly affects large synovial joints such as the knee and the elbow and the main clinical symptoms include fever, maculopapular rash and joint and muscle pain. The high viral load is initially controlled by the host innate immune response, while later during the infection it correlates with the development of neutralizing IgM and IgG antibodies. Despite the robust host immune response, viral particles can persist in the synovial fluid of the joints, which can result in severe pathology lasting months to years. Reused with permission from (90).

Current treatment and a historical perspective on CHIKV antiviral development

Treatment for CHIKF relies on supportive care, short-term opioid analgesics, paracetamol or acetaminophen, and non-steroidal anti-inflammatory drugs (NSAIDs). However, NSAIDs need to be prescribed with caution in patients with a suspected DENV infection, given the possible haemorrhagic complications (91). As the pathogenesis of CCA is not well understood, treatment of patients has remained empirical. In the absence of evidence-based treatment, clinicians often prescribe a variety of anti-inflammatory drugs, in addition to symptomatic treatment with

NSAIDs. Such inflammation suppressing agents include corticosteroids, chloroquine, hydroxychloroquine, sulfasalazine, methotrexate and immune-modulating therapies targeting proinflammatory mediators or T cell responses (reviewed in (92, 93)). So far only three trials examined a therapeutic approach to treat CHIKF in humans. In the early 1980s, a first pilot study to evaluate the efficacy of a potential CHIKV treatment using chloroquine phosphate was conducted. The 20-week treatment program of a small cohort of 10 patients with a daily dose of 250 mg improved the condition in 7 out of the 10 patients (94). In 2007, however, a study on La Reunion Island disproved the benefits of chloroquine treatment on relieving CHIKV symptoms (95). Chloroquine was also tested in an exploratory intervention trial against a commonly used NSAID meloxicam, but failed to prove efficacy in treating musculoskeletal pain and arthritis in patients with acute CHIKV infection (96). The current stages of CHIKV small-molecule antiviral drug development will be discussed in Chapter 2.

Non-small molecule-based inhibitors: treatment with monoclonal (m) Abs and nucleic acids

Besides small-molecule inhibitors, treatment with mAbs has been used as a powerful strategy for the short-term treatment of emerging viral diseases. Neutralizing CHIKV mAbs can block CHIKV entry, fusion and release. The advantage of passive immunization is that mAbs can be administered to any age, high-risk or immunocompromised population due to their safety. However, the high cost associated with the manufacturing of recombinant mAbs represents the biggest obstacle for their development. Several potent human and mouse anti-CHIKV mAbs were shown to be protective against CHIKV *in vivo* (reviewed in (97)). They can be given prophylactically or administered post-exposure to limit CHIKV spread and reduce symptoms in infected individuals. More research is needed to investigate the ability of mAbs to prevent CHIKV persistence in joint tissues. Given the high mutation rate of alphaviruses, escape mutants can develop following treatment with mAbs during acute phase, a problem that can also be encountered during treatment with small-molecule inhibitors. This problem may be overcome by administration of a combination of mAbs targeting different epitopes. One study showed that escape mutants from combination antibody therapy are clinically attenuated (98), arguing for the use of combination therapy in preventing the emergence of drug resistance. Modern approaches rely on the injection of nucleic acids (DNA or RNA) encoding Ab sequences for *in vivo* production of recombinant Abs.

These approaches obviate the need for complex manufacturing and save costs. For instance, a study describing the use of CHKV24 mRNA-encoded mAb formulated in a lipid nanoparticle for treatment of CHIKV-infected mice and NHPs reported protection against arthritis and musculoskeletal disease (99).

CHIKV vaccine development

CHIKV vaccines have been in development for over 50 years, but the efforts intensified only in the past decade with a total of eight promising candidates in Phase I, Phase II and one vaccine candidate currently entering Phase III clinical trials. Considering that CHIKV antigenic diversity is limited and infection may lead to long-lasting immunity, developing a CHIKV vaccine seems to be particularly attractive. Importantly, reciprocal cross-protection after infection with other alphaviruses such as MAYV (100) or ONNV (101) has been reported in animal models, potentially increasing the success of such a vaccine in low-income countries with a high disease burden. Nevertheless, a licensed vaccine against CHIKV is not available thus far. The global market for a CHIKV vaccine has been estimated to be approximately €500 million annually (17). For comparison, the global influenza vaccine market today has on average still a ten-fold higher market value.

Researchers have used various platforms to develop a CHIKV vaccine, including inactivated viral vaccines, live-attenuated virus (LAV) vaccines, recombinant viral-vectored vaccines, virus-like particle (VLP) vaccines, subunit vaccines and nucleic acid-based vaccines. Several CHIKV LAV vaccine candidates have been produced with either mutations, deletions or modifications in CHIKV nonstructural or structural proteins (102-108). In general, LAV vaccines can stimulate stronger and longer-lasting immune response at the cost of lower safety due to the risk of reversion to virulence.

The first CHIKV vaccine tested in volunteers, an isolate from a patient in Thailand named CHIKV strain AF15561, was formalin-inactivated and developed by scientists at the Walter Reed Army Institute of Research, USA (109). An attenuated CHIKV clone selected by passaging strain AF15561 18-times in MRC-5 cells, called CHIKV 181/clone 25 (110), was then tested for safety and immunogenicity in a Phase I trial and entered Phase II clinical trials in 2000 (111). The development of this LAV vaccine was discontinued due to virus-specific side effects, including arthralgia, in approximately 8% of the vaccinees and due to the low incidence of the disease at the time. Studies in mice indicated that the attenuation of CHIKV vaccine strain 181/25 relies on two

mutations in the E2 glycoprotein T12I and G82R (112). Mechanistically, the mutation G82R likely promotes the interaction of the positively charged amino acids within E2 with the negatively charged GAGs (113).

In CHIKV recombinant virus-vectored vaccine development, measles vector has been widely used as a replicating vector owing to its remarkable safety and efficacy. A live-attenuated recombinant viral vectored vaccine comprising the Schwarz vaccine strain of measles virus (MV) engineered to express CHIKV structural proteins has recently been announced to progress into Phase III clinical trials. The CHIKV RNA sequence in this vaccine is derived from strain 06-49 belonging to the ECSA lineage, which was isolated from a patient during the La Reunion outbreak. This vaccine, MV-CHIK, is highly immunogenic and protects mice (114) and NHPs (115) from lethal challenge with CHIKV. In a randomised, double-blind, placebo-controlled Phase I trial the MV-CHIK induced highly neutralizing anti-CHIKV Abs in healthy adults, even in the presence of pre-existing vector immunity (116). After two immunisations, the patients reached a 100 % seroconversion rate. The MV-CHIK vaccine also elicits cross-neutralizing immune response to the CHIKV vaccine strain 181/25, which is based on an Asian lineage isolate. In a randomised, double-blind, placebo-controlled and active-controlled Phase II trial conducted in Europe, healthy volunteers aged 18-55 years were subjected to a dose of control vaccine, MV-CHIK or measles prime and MV-CHIK via the intramuscular route (ClinicalTrials.gov Identifier: NCT02861586). MV-CHIK demonstrated an excellent safety and tolerability in this trial (117) and became the first CHIKV vaccine to enter a Phase III clinical trial.

Another candidate vaccine that entered Phase II clinical trials consists of non-replicating chikungunya VLPs developed at the National Institutes of Health, USA. The VLP vaccine (VRC-CHKVLP059-00-VP) elicited high titre of neutralizing anti-CHIKV Abs in a NHP model of CHIKV infection and protected from disease (118). In a Phase I study with healthy volunteers, the vaccine was well-tolerated and immunogenic, with a second booster vaccination reaching a 100 % seroconversion rate, similar to the MV-CHIK vaccine (119). In a Phase II clinical trial conducted among healthy adults aged 18-60 years in Central America, the study volunteers were subjected to two intramuscular injections given 28 days apart and followed for 72 days (ClinicalTrails.gov Identifier: NCT02562482). The VLP vaccine was well tolerated and demonstrated good safety (120). There is good evidence that a vaccine made against one genotype elicits cross-protective immune response against all CHIKV genotypes, as demonstrated by a study in which recipients of the VLP vaccine developed cross-reactive neutralizing anti-

CHIKV Abs to 9 CHIKV strains belonging to all 3 genotypes (121). On the basis of the promising Phase II results, both MV-CHIKV and CHIKV VLP vaccines have been granted the priority medicines status by the European Medicines Agency.

Vector control strategies

The global population at risk of mosquito-borne infections such as CHIKV, DENV or ZIKV is expanding due to changes in distribution of two key vectors: *Ae. aegypti* and *Ae. albopictus.* Their worldwide distribution is largely driven by the presence of suitable climate and human movement. Until a vaccine or therapeutic treatment for CHIKF becomes available, people living in the world's most affected areas have to rely on vector control to limit the contact with virus-carrying mosquitoes. Traditional methods in controlling vector-borne diseases use larvicides and insecticides, but their use has become less effective due to the emergence of insecticide resistance and the inability of insecticides to penetrate house walls, where many adult female mosquitos rest and feed (6). Modern strategies of mosquito control include the Sterile Insect Technique to generate transgenic sterile male *Ae. aegypti* mosquitoes. This lethality-based approach essentially reduces the mosquito population and, as such, possibilities for viral transmission (122). More elegant strategies have used a maternally-inherited bacterial endosymbiont called *Wolbachia*, which has a strain-specific effect. For example, *Ae. aegypti* mosquitoes transinfected with the wMel and wMelPop strains resulted in reduced CHIKV transmission (123, 124). On the other hand, *Ae. albopictus* is a natural carrier of two strains of *Wolbachia*, wAlbA and wAlbB, which have no effect on CHIKV infection (125). Interestingly, when the wMel strain was introduced into the *Ae. albopictus* mosquitoes in the presence of the natural strains, reduction of CHIKV transmission was observed (126). Together with other mitigation strategies such as behavioural and protective measures, including wearing long-sleeved clothing, vector control has become crucial in diminishing the impact of CHIKV infection on human lives in affected areas. However, mosquito control programs are not highly efficient in outbreak containment. Antiviral treatment represents the most effective control measure during outbreak situations as will be discussed in Chapter 2.

Thesis outline

This thesis describes the identification and characterization of several new classes of CHIKV nsP1-targeting inhibitors. It begins by describing the selection of antiviral compounds through rational drug design and antiviral drug screening, proceeds with detailed molecular *in vitro* studies to identify the viral target of selected compounds and concludes with molecular docking studies on the CHIKV nsP1 cryo-EM structure.

Chapter 2 contains a comprehensive literature review on CHIKV small-molecule inhibitors. It further discusses relevant topics in modern CHIKV antiviral drug discovery including the choice of cell lines and animal models for CHIKV antiviral research.

Chapter 3 describes the identification of 6'-fluorinated-aristeromycin and 6'- fluorinated-homoaristeromycin analogues as novel CHIKV inhibitors. The ability of these compounds to inhibit CHIKV cytopathic effect, reduce viral load and their cytotoxicity in mammalian cells are reported.

Chapter 4 represents a mode of action study of 6′-β-fluoro-homoaristeromycin and 6-fluoro-homoneplanocin A in CHIKV-infected cells. It describes the identification of CHIKV nsP1 as the target of these antiviral compounds in cell-based assays and *in vitro* enzymatic assays with purified SFV nsP1.

Chapter 5 is a mode of action study of a novel class of CHIKV small-molecule inhibitors called CHVB. It characterizes the antiviral effect of these compounds on several CHIKV clinical isolates and identifies CHIKV nsP1 as the antiviral target of the CHVB series. Antiviral activity is confirmed using *in vitro* enzymatic assays with VEEV and SFV nsP1.

Chapter 6 explores the relationships between CHIKV nsP1 mutants that are resistant to different classes of nsP1-targeting compounds. It describes molecular docking of selected CHIKV nsP1 inhibitors on a recently solved CHIKV nsP1 cryo-EM structure and provides interpretations with regard to their mode of action.

Chapter 7 discusses the key findings of this thesis in a broader context and emphasises their implications for future CHIKV antiviral research.

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