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Defining a correlate of protection for chikungunya virus vaccines

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ABSTRACT

Chikungunya virus infection causes a debilitating febrile illness that in many affected individuals is associated with long-term sequelae that can persist for months or years. Over the past decade a large number of candidate vaccines have been developed, several of which have now entered clinical trials. The rapid and sporadic nature of chikungunya outbreaks poses challenges for planning of large clinical efficacy trials suggesting that licensure of chikungunya vaccines may utilize non-traditional approval pathways based on identification of immunological endpoint(s) predictive of clinical benefit. This report reviews the current status of nonclinical and clinical testing and potential challenges for defining a suitable surrogate or correlate of protection.

1. Introduction

Chikungunya fever (CHIKF) is a non-specific febrile illness caused by infection with chikungunya virus (CHIKV) that is often characterized by high fever, rash, headache and myalgia, with a high frequency of acute and chronic polyarthralgia/polyarthritis that can be severely debilitating and long-lasting [1,2]. Although rare, other severe and potentially fatal outcomes of CHIKV infection can occur - including neurological, ocular or cardiovascular manifestations, hepatitis, and hemorrhage - and are typically more prevalent in very young or old subjects or are associated with other comorbidities [3]. Lethal or severely disabling neurological disease following vertical transmission of CHIKV has also been reported to occur at high frequency in neonates whose mothers are viremic at the time of delivery [4].

Although CHIKF has a very low overall case fatality rate (CFR) the burden of disease during and following epidemics is significant [5], suggesting that development of a prophylactic vaccine may be of benefit for use in outbreak response or for travelers to CHIKV endemic areas. CHIKV infections cause rapidly developing, large epidemics with up to 50–70% of the population infected. Incidence of new infections, however, declines rapidly following those epidemics due to the high infection rates and subsequent development of herd immunity, which makes implementation of large clinical efficacy trials extremely challenging. Therefore, the licensure of chikungunya vaccines on the basis of immunological markers of efficacy via either traditional or non-traditional regulatory pathways must be considered.

The potential pathways and regulatory considerations for licensure of chikungunya vaccines by the United States Food and Drug Administration (FDA) have recently been discussed in detail [6]. These include the possibility for traditional licensure in the basis of clinical immunogenicity assessment using a robust, mechanistic correlate of protection (CoP; i.e. immune response(s) that is/are directly linked to protection against CHIKV infections, or CHIKF disease, in a causative way). Among currently licensed vaccines, a well-established CoP exists for Influenza A vaccines, for example, which is neutralizing antibody responses against the viral hemagglutinin (HA) protein [7]. A hemagglutination inhibition (HI) titer of over 1/40 is an acceptable measure to ensure at least 50% protection, which makes it a relative CoP and indicates that other immune responses contribute to protection [8,9]. Alternatively, the FDA may consider licensure of chikungunya vaccines via the "accelerated approval" pathway, based on clinical assessment of a surrogate endpoint, such as an immunological marker, that is "reasonably likely" to predict clinical benefit in humans [6]. Similarly, in the absence of robust clinical efficacy data for a candidate vaccine, the European Medicines Agency (EMA) may consider Conditional Marketing Authorization which was established for

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products that address unmet medical needs that include (among other categories) seriously debilitating diseases or are intended for use in emergency situations [10], for which chikungunya vaccines may qualify.

For the purpose of this report, immunological markers will be discussed that may serve as surrogate endpoints or as mechanistic CoPs, as defined by Yang et al. [6]. For CHIKV, *in vitro* and *in vivo* studies have shown that neutralizing antibodies are a major effector of protection against CHIKV infection and disease, but a significant amount of work remains to be done to facilitate definition of a robust CoP.

2. Chikungunya virus

CHIKV is an alphavirus (Family *Togaviridae*, genus *Alphavirus*) whose genome is a single stranded, positive sense RNA of approximately 11.8 kb that encodes nine viral proteins in two open reading frames, with the structural proteins encoded in a subgenomic region in the 3′ third of the genome. The mature CHIKV virion is comprised of the viral genome packaged in a nucleocapsid core surrounded by a lipid and protein envelope in which the viral E1 and E2 proteins are present in heterodimers [11]. The E2 protein mediates virus attachment to yet-to-be-defined cell surface receptor(s), while the E1 protein facilitates fusion of the viral membrane with the host cell membrane under acidic conditions. Transmission of CHIKV in nature typically involves enzootic (or sylvatic) maintenance of the virus in cycles amongst nonhuman primate and possibly other vertebrate animal hosts by various *Aedes* spp. mosquitoes [12]. CHIKV epidemics are associated with human-to-human transmission of the virus by *Ae. aegypti* and *Ae. albopictus*.

Molecular phylogenetic analyses of CHIKV isolates have defined three distinct genotypes – West African, East/Central/South African (ECSA), and Asian [12]. Despite the description of multiple genotypes, CHIKV strains are generally accepted to constitute a single serotype and, although strain/genotype-specific differences in antigenicity and neutralization have been reported, these are relatively limited and seem unlikely to impact cross-protective capacity of CHIKV vaccine candidates [13,14]. Beginning in 2004, a large outbreak in the Indian Ocean region was associated with the ECSA lineage and the emergence of a distinct subtype, described as the Indian Ocean lineage (IOL), which was demonstrated to encode adaptive mutations that facilitated transmission of the virus by *Ae. albopictus* mosquitoes [12]. Between 2004 and 2009, more than 2 million cases of CHIKF were estimated to have occurred throughout the Indian Ocean area associated with large outbreaks in the Union of Comoros, La Reunion Island and in southern India, and resulting in introduction of the virus to many other areas by travelers, including the Americas and Europe. In most cases, local spread did not occur or was limited, although almost 200 cases occurred in Italy following introductions of CHIKV. In 2014, CHIKV emerged in the Americas and caused a large epidemic affecting most of Central and South America and the Caribbean, with almost 2.5 million cases estimated to have occurred during 2014–16 [15]. Somewhat surprisingly, this outbreak was associated with the Asian genotype of CHIKV, although isolates of the ECSA genotype were also detected in one region of Brazil [16].

During 2017, CHIKV transmission in the Americas continued to decline, although more than 123,000 CHIKF cases were reported [17], primarily from Brazil. Pakistan has experienced its first significant outbreak with more than 3000 suspected cases in the large city of Karachi and from several other provinces [18,19], a small outbreak occurred during July-August in Cannet-des-Maures in the south of France with confirmed infections of six individuals, all living in the same neighborhood and with no travel history [20,21], and more than 200 confirmed cases were also reported during the second half of 2017 associated with an outbreak in the Lazio and Calabria regions of Italy [22,23].

3. Immunology of CHIKV infections and basis of recovery/protection

Innate immune responses arise very early after CHIKV infection and various innate components play roles in limiting the infection and in pathogenesis of disease in joint and muscle tissue. CHIKV is sensitive to type 1 interferons and vigorous type I interferon responses are induced following CHIKV infection [24]. Stimulation of the type I interferon receptor pathway results in activation of interferon-stimulated genes that are critical for early control of CHIKV [25,26]. The innate cellular response to CHIKV infection apparently involves mobilization of a number of innate cell types including macrophages, dendritic cells, basophils, neutrophils and eosinophils. Viremia associated with CHIKV infection usually resolves within three weeks of infection; however, many patients develop chronic infection with associated arthropathies at later time points. As the infection progresses, viral antigens have been detected in muscle and joint-associated tissues concomitant with large mononuclear cell infiltrates [27]. Macrophages, monocytes and natural killer (NK) cells usually predominate the innate cellular response and have been detected in synovial exudates from patients infected with CHIKV or the related alphavirus, Ross River virus (RRV) [28–30]. While abundant in synovial tissues following CHIKV infection, the exact mechanisms underlying protective and/or pathogenic roles for these cells are still being completely defined. Mice depleted of macrophages by treatment with chlondronate liposomes displayed increased viremia and decreased disease measured as diminished foot swelling suggesting both protective and pathogenic roles for macrophages in CHIKV infection [27]. Macrophages have been shown to be susceptible to CHIKV infection and may aid in dissemination of virus. Additionally, macrophages may contribute to CHIKV pathogenesis by migrating to infected synovial tissues and participating in the chronic inflammatory response and serve as a cellular reservoir for persistent CHIKV infection [30,31].

A number of inflammatory cytokines are significantly upregulated during CHIKV infection. Serum cytokines detected at increased levels from a group of 69 acutely infected patients during the 2007 Gabonese outbreak included IL-6, IL-7, IL-12p40, IL-16, IL-17, IP-10, MCP-1, MIF, SDF-1α, IL-1Rα, IL-2Rα, G-CSF, GM-CSF, VEGF, and IFN-α2 [32]. In other clinical studies, high levels of IL-1β and IL-6 were identified as biomarkers associated with severe CHIKF disease [24]. Robust inflammatory cytokine responses can be maintained throughout persistent infection with increased levels of IL-6 and GM-CSF detected in CHIKV infected humans with persistent arthralgia [33] and vigorous MCP-1, MIP-1α, and MIP-1β chemokine responses during chronic CHIKV infection [34]. Interestingly, there appears to be a similarity in expression of inflammatory cytokines and immune mediators expressed in the CHIKV arthritis model and rheumatoid arthritis suggesting similarities in the inflammatory nature of these diseases [35].

3.1. Adaptive immunity to CHIKV

Cell-mediated responses to CHIKV infection have been characterized in both animal models and from human CHIKV infection. In CHIKV-infected humans, an increase in the CD8+T cell response is noted in the first days after onset of symptoms. Of blood samples analyzed following the 2007 Gabonese outbreak, a higher percentage of CD3+CD8+T cells was detected in CHIKV infected patients relative to controls during the first three days after onset of

symptoms [32]. The T cell response of humans apparently targets several CHIKV proteins. Analysis of the protein-specificity of CHIKV-specific T cell response from 48 patients infected by CHIKV during the 2005–2006 La Reunion Island outbreak by ELISPOT, proliferation response, and intracellular cytokine staining revealed T cells specific for the capsid, E2, and nsP1 proteins. A hierarchy of response was observed with the majority of T cells recognizing the E2 protein followed by the nsP1 protein and finally the capsid protein. Interestingly, the T cell responses to these proteins were of identical strength in patients that had fully recovered compared to those experiencing chronic infection [36].

The role of the cell-mediated arm of the adaptive immune response in virus clearance and protection against alphaviruses is incompletely understood and appears dependent on variables such as the virus type and tissue infected. Adoptively transferred VEEV-primed CD4+T cells, but not transferred antibody, prevented development of encephalitis in T cell receptor deficient mice [37]. Evidence for clearance of RRV by CD8+T cells was observed in the skeletal muscle, but not joints of infected animals [38] whereas in other studies, vaccine-induced RRV-specific CD8+T cells had no impact on viremia [39]. Similarly, in a murine model of CHIKV infection, depletion of T cells from CHIKV vaccine-immune mice or transfer of CHIKV-primed T cells did not protect against mortality. However, passive transfer of immune serum with a minimum neutralizing titer of 35 was sufficient to protect recipient mice against disease [40].

Evidence from mouse models suggests that the adaptive immune response may modulate joint pathogenesis although more research is required to determine the exact cells and molecular mechanisms involved. Inoculation of wild-type and Rag 1−/− mice, deficient in adaptive immune cells, with CHIKV resulted in a chronic infection characterized by the persistence of CHIKV RNA in joint-associated tissues and development of synovitis, arthritis, and tendonitis. Because the arthritis detected in Rag 1−/− mice at day 7 post infection was of lower intensity than in wild type mice, it was suggested that the adaptive immune response may play a role in the development of early joint disease [41]. In addition to innate immune cells, CD4+and CD8+T lymphocytes are readily detected in infected joint and muscle tissue in murine models of CHIKV and RRV infection [27,42,43]. In studies utilizing genetic knockout mice, inoculation of wild type mice with CHIKV resulted in significant joint swelling. While CHIKV infected CD4−/− mice cleared virus similarly to wild type mice, joint disease was not observed. By contrast, CHIKV infected CD8−/− mice also cleared virus, but developed joint swelling suggesting that CD4+, but not CD8+T cells were essential for the development of joint pathogenesis [44]. Consistent with these findings, CD4+ and CD8+T cells were detected in the joints tendons, and skeletal muscle of 14 day old mice inoculated with CHIKV [42] and numerous CD4+, but only rare CD8+T cells were found in the synovia of chronically infected CHIKV patients from the 2006–2007 outbreaks in La Reunion [30].

CHIKV-specific antibodies have been shown to play an important role in protection against CHIKV and the cumulative evidence suggests that neutralizing antibody likely represents a correlate of protection. Initial studies to define the role of antibodies in experimental animals utilized CHIKV infection of B cell deficient μMT mice. Wild type controls cleared the viremia, whereas CHIKV infected μMT mice exhibited a long-persisting viremia and more severe joint disease indicating a role for B cells in resolution of viremia and modulation of disease [45]. Serum from CHIKV-infected wild type mice contained neutralizing antibodies directed primarily at the E2 protein. Passive transfer of IgG antibodies from plasma of convalescent patients can efficiently prevent and cure CHIKV infection in mice [46,47]. Additionally, treatment with neutralizing monoclonal antibodies (mAbs) specific for CHIKV E1 and E2 proteins protected IFNαβR−/− mice against mortality [48] and prevented development of chronic infection of Rag-1−/− mice when given up to 3 days post infection. Interestingly, treatment at three weeks post infection apparently eliminated infectious CHIKV from sera and quadriceps muscle, but had little effect on CHIKV RNA burdens in joint-associated tissue [41]. Although the human mAbs 8B10 and 5F10 prolonged survival, they did not prevent lethality in mice [49,50]. Other human mAbs however, like mAb C9 [51], or mAbs 4 J21 and 5 M16 [52] were protective in a lethal challenge model in immunocompromised mice. This indicates that neutralizing antibodies alone might be sufficient for protection.

In humans, the presence of CHIKV-neutralizing antibodies prior to infection was shown to modulate disease. Baseline and convalescent serum samples were obtained during a prospective study of individuals with acute febrile illnesses between 2012 and 2013 in Cebu City, Philippines [53]. Symptomatic and asymptomatic individuals were identified using CHIKV PCR of acute blood and/or CHIKV IgM/IgG ELISA seroconversion in paired acute/convalescent samples. An anti-CHIKV PRNT titer of greater than 10 was associated with protection from development of symptomatic CHIKV infection and was identified as a potential immune CoP [53].

The human CHIKV-specific antibody response resulting from CHIKV infection has been characterized in several studies. Like the response in experimental animals [45], human antibodies target mainly to the E2 glycoprotein [54,55]. Envelope-specific antibodies from serum collected between days 7 and 15 after infection from CHIKV patients in Bangalore, India were comprised predominantly of IgG3 subclass antibodies [55]. In a study of serum antibodies from the 2008 Singapore outbreak, the early development of a neutralizing antibody response of the IgG3 subclass was associated with CHIKV clearance and clinical protection against persistent arthralgia, whereas patients with delayed IgG3 responses were more likely to experience chronic joint pain [47,56], suggesting that antibodies of this type may be a useful CoP. Similar findings regarding the importance of early neutralizing antibody responses in protection against arthralgic complications of CHIKF disease have also been recently reported from analysis of a prospective cohort of CHIKF patients in India [57]. Because IL-6 promotes B cell growth and shifts to IgG3 subclass expression [58], it is thought that the high levels of IL-6 that are commonly detected following CHIKV infection [24,33] may be responsible for development of the IgG3 response.

Given the abundant evidence for the critical role of CHIKV-specific neutralizing antibodies to protect against mortality, development of disease, and clearance of viremia, and the paucity of evidence for protection by T cell mediated immunity, it is likely that effective vaccines will need to elicit a strong, durable, neutralizing antibody response. Such a response will also require induction of a CD4+T helper cell response. Although there is evidence that CD4+T cells can play a role in increasing pathogenesis of joint-associated tissue [44], the exact role played by this subset and the molecular mechanism underlying the pathogenesis is not understood. The development of only asymptomatic CHIKV infection in the presence of a threshold level of neutralizing antibody [53] suggests that high levels of vaccine-elicited antibody may mask or prevent this potential CD4+T cell mediated pathogenic effect.

3.2. Potential for long-lived immunity to CHIKV

Evidence for persisting immunity to CHIKV is found in the analyses of serum antibodies from previously infected humans. For example, the serum antibody response of a cohort of patients from the 2007 Italy CHIKV outbreak was followed after the acute phase of infection. CHIKV-specific IgM and IgG or IgG-only antibodies were detected in the serum 12 months after the acute phase of

Table 1
Current chikungunya vaccine candidates in clinical trials or with data reported from studies in nonhuman primates.

Vaccine type/description	References	Clinical Trial identifier(s)[a]
Virus-like particles (VLPs)	Akahata et al., 2010 [101]; Chang et al., 2014 [102]; Goo et al., 2016 [14]	NCT01489358; NCT02562482; NCT03483961
Recombinant, Attenuated Measles Virus expressing CHIKV VLPs	Brandler et al., 2013 [88]; Ramsauer et al., 2015 [89]	NCT02861586; NCT03101111; NCT03028441; 2015-004037-26
Attenuated CHIKV with Truncation of 3′ End of nsP3 Coding Sequence	Hallengärd et al., 2014a [83]; Roques et al., 2017 [105]	NCT03382964
mRNA-based vaccine for VLP expression	N/A	NCT03325075
Formalin inactivated whole virus	N/A	CTRI/2017/02/007755
DNA vaccine plasmid containing E3, E2, and E1 proteins from CHIKV	Mallilankaramen et al., 2011 [65]	N/A
Attenuated CHIKV containing IRES from ECMV	Plante et al., 2011 [81]; Roy et al., 2014 [110]	N/A
Chimeric Eilat Virus expressing CHIKV structural proteins	Erasmus et al., 2017 [85]	N/A

[a] Trials identified with "NCT" numbers are listed in the U.S. National Library of Medicine ClinicalTrials.gov database (https://clinicaltrials.gov/). Details of the "CTRI" trial are listed in Clinical Trials Registry – India (http://ctri.nic.in/Clinicaltrials/advancesearchmain.php/). Trial 2015-004037-26 is listed in the EU Clinical Trials Register (https://www.clinicaltrialsregister.eu/).

infection [59]. In another study, serum antibody from nine patients originally admitted for acute CHIKV infection during the 2008 Singapore outbreak was collected at 21 months post illness onset. Although at lower levels than samples taken 2–3 months post illness onset, CHIKV-specific IgG with neutralizing activity was demonstrated in serum samples of all patients [54]. Epidemiological evidence obtained during the 2011–2012 outbreak involving the ECSA genotype of CHIKV in Trapeang Roka, Cambodia suggests persistence of CHIKV antibody responses of approximately 40 years. During the outbreak, it was established by questionnaire and infection confirmation by the presence of CHIKV IgM that individuals over 40 years of age experienced the least risk of infection. Severely restricted travel in Cambodia during the Khmer Rouge regime beginning in 1975 impacted circulation of CHIKV and outbreaks were apparently undetectable in Cambodia through 2011. Importantly, the presence of pre-existing, cross-protective antibody in the over-40 population, acquired as a result of CHIKV outbreaks in the 1960′s was thought most likely responsible for the protection during the ECSA outbreak [60]. Similar long-term persistence of neutralizing anti-CHIKV antibodies has also been reported in a significant subset (>40%) of affected individuals almost 20 years following a CHIKV outbreak in Thailand [61].

4. Vaccine development for chikungunya

The history and current status of vaccine development for CHIKF have been extensively reviewed in recent years [5,62–64]. Table 1 provides a brief summary of current candidates for which data from clinical trials or studies in nonhuman primates have been reported, and a Supplementary Table provides an overview of many vaccine candidates described in the literature and the approaches employed to characterize immune responses, particularly neutralizing antibody responses, stimulated by those products in animal models and/or human recipients. Development of candidate vaccines to prevent CHIKV infection and disease dates to the mid-20th century when inactivated and live attenuated vaccine candidates were developed by the United States military. Since the mid-2000s, a large number of candidate vaccines based on a wide variety of platforms have undergone nonclinical evaluation to demonstrate induction of protective immunity in small animal and/or nonhuman primate models, with a small number of those progressing to phase I/II clinical trials. Consistent with the perceived importance of neutralizing antibodies in protection against and resolution of CHIKV infections, the vast majority of vaccine candidates have focused on induction of neutralizing antibodies against the viral structural proteins. The E2 protein, which is the primary receptor binding protein, is the main target for neutralizing antibodies, although studies have indicated that induction of robust neutralizing antibody responses is generally improved by presentation of E1 and E2 proteins in native virion or virus-like particle (VLP) structures [e.g. 65,66]. In particular, cryo-electron microscopy studies have identified potently and/or broadly cross-reactive neutralizing antibodies that appear to recognize epitopes that bridge across multiple proteins in the native CHIKV virion structure [67,68].

Immunogens employed for CHIKV vaccine development have included inactivated virus (purified or in crude tissue culture supernatants) [66,69–78]; live, attenuated CHIKV strains derived via empirical cell culture adaptation [79,80] or rational genetic modification [81–84]; live, chimeric vectored vaccines based on heterologous alphavirus backbones [85–87] or other viral vectors including measles [88,89], VSV [90], or vaccinia [91–91] expressing CHIKV structural proteins; DNA vaccines [65,94,95] expressing one or more CHIKV structural proteins [65,96,96], a CHIKV replicon subgenome [97] or an entire live, attenuated CHIKV genome [95]; virus-like particles expressed and purified from insect [98,99], yeast [100] or mammalian cells [14,101,102]; and recombinant proteins [66,103,104]. In addition, DNA vaccination with concurrent expression of a CHIKV-neutralizing monoclonal antibody has been described [96]. Some recent studies have also investigated heterologous prime/boost strategies using combinations of candidates, including attenuated, DNA and VLP vaccines in mice and NHPs [97,105].

Assessments of vaccine immunogenicity for these candidate vaccines have employed a wide variety of assay types (Table 2) and Supplementary Table. Levels of anti-CHIKV IgG antibody, and in some cases IgM and/or IgA, have been assessed by standard indirect ELISAs using infected cell lysate antigens [106], purified and inactivated CHIKV [66,75,76,82,93,98], synthetic peptides [94], or recombinant CHIKV structural proteins [66,83,88,91,94,103,104] or VLPs [100,102]. Antigens employed for ELISAs are generally homologous to the CHIKV strain utilized for development of the particular vaccine candidate. Neutralizing antibody responses have been measured via standard plaque reduction neutralization assays or microneutralization assays on Vero or BHK cells using a range of CHIKV strains, and some studies have included comparisons against multiple CHIKV strains to begin to assess any potential for vaccine failure/escape [14]. However, specific assay conditions used to assess neutralizing antibody levels vary greatly between studies (Supplementary Table) and the potential impacts of those technical differences on comparability of the data is

Table 2
Immunoassays for quantification of anti-CHIKV antibody responses induced by candidate vaccines.

Assay type	Antigen	Endpoint(s)[a]	Reference(s)
Indirect ELISA (primarily IgG and IgG subclasses; also IgM and/or IgA)	CHIKV antigen (inactivated or cell lysate); or chimeric alphavirus with CHIKV structural proteins	Titer; or OD at single dilution (typically 1:100)	McClain 1998 [106]; Tiwari et al., 2009 [74]; Prow et al., 2010 [75]; Wang et al., 2011 [118]; Kumar et al., 2012 [66]; Chattopadhyay et al., 2013 [90]; Piper et al., 2013 [82]; Roy et al., 2014 [110]; Weger-Lucarelli et al., 2014 [93]; Weber et al., 2015 [104]; Rudd et al., 2015 [76]
	Virus-like particle (VLP); expressed from mammalian or yeast cells	Titer	**Chang et al., 2014**[b] [102]; Saraswat et al., 2016 [100]
	E1/E2 synthetic peptides	OD at 1:100 dilution	Muthumani et al., 2008 [94]
	Recombinant E1 and/or E2 protein (expressed from bacteria or insect cells)	Titer	Khan et al., 2012 [103]; Kumar et al., 2012 [66]; Brandler et al., 2013 [88]; Metz et al., 2013a/b [98,99]; Hallengard et al., 2014 [83,97]; Roques et al., 2017 [105]
Microneutralization	WT or infectious clone-derived CHIKV strains	Inhibition of CPE in 50–100% of replicate wells; in Vero/BHK	Mallilankaraman et al., 2011 [65]; Wang et al., 2011 [118]; Kumar et al., 2012 [66]; Metz et al., 2013a/b [98,99]; van den Doel et al., 2014 [92]; Weger-Lucarelli et al., 2014 [93]
Plaque/focus reduction neutralization	WT or infectious clone-derived CHIKV strains; 181/25 live, attenuated vaccine strain	50–90% reduction in plaque/ immunofocus numbers; in Vero/BHK	Tiwari et al., 2009 [74]; Plante et al., 2011 [81]; Wang et al., 2011 [86]; Khan et al., 2012 [103]; Piper et al., 2013 [82]; Brandler et al., 2013 [88]; Roy et al., 2014 [110]; Tretyakova et al., 2014 [95]; **Ramsauer., et al. 2015** [89]; Saraswat et al., 2016 [100]; Erasmus et al., 2017 [85]
Reporter virus neutralization	Recombinant CHIKV or chimeric alphavirus (with CHIKV structural proteins), expressing GFP.	50–90% reduction in reduction in number of fluorescent cells (by microscopy or flow cytometry); in HEK 293T, Vero.	**Chang et al., 2014** [102]; **Goo et al., 2016** [14]; Taylor et al., 2017 [119]
	Recombinant CHIKV packaged replicon expressing luciferase	50% reduction in luciferase activity; in BHK-21	Glasker et al., 2013 [108]; Garcia-Arriaza et al., 2014 [91]; Roques et al., 2017 [105]
	Lentivirus CHIKV pseudotypes, expressing luciferase	Reduction in luciferase activity; in HEK 293T, 293A, Vero	Akahata et al., 2010 [101]; Weber et al., 2015 [104]
	VSV CHIKV pseudotypes expressing GFP	Inhibition of GFP production in Vero cells, assessed by microscopy	Chattopadhyay et al., 2013 [90]

[a] For ELISAs, various cutoffs for determining endpoint dilutions have been used e.g. two standard deviations above, or two times, the mean absorbance from negative control sera, blank wells, or pre-immune sera from the same individual.
[b] Bolded references are for clinical trials.

currently uncertain. Due to the relatively low-throughput nature of standard neutralization assays and the requirement for BSL3 containment for wild-type CHIKV strains, alternative neutralization methods have been developed that include use of lentiviral CHIKV pseudotypes with a luciferase reporter [101,104,107], CHIKV packaged replicons with a luciferase reporter [83,108], or a flow cytometry-based assay using a GFP-expressing CHIKV/Sindbis virus chimera [48,102]. Development of these alternative assays has generally included comparisons using limited numbers of representative polyclonal sera and/or monoclonal antibodies to benchmark their performance against standard PRNT methods using live CHIKV. At the current time, standard/control antibodies and antisera utilized in ELISA or neutralization assays supporting advanced development of leading CHIKV vaccine candidates are "in house" reagents prepared or sourced by the vaccine developers (personal communication to DWCB). Many of the early CHIKV vaccine development studies employed complement fixation (CF) and hemagglutination inhibition (HI) tests as alternative measures of CHIKV-reactive antibody titers, and HI titers have also been measured in one recent clinical trial [89]. However, both CF and HI are also technically complicated assays to perform on a high-throughput basis.

Immunocompetent (e.g. C57BL/6, BALB/c) and immunodeficient mice (e.g. A129, IFNAR) have typically been used as small animal models to assess immunogenicity and protective efficacy of CHIKV vaccine candidates (Table 3, Supplementary Table). In adult immunocompetent mice, peripheral inoculation with CHIKV strains causes no or few clinical signs, so efficacy endpoints are typically assessment of viremia, weight loss, and/or footpad swelling [75,76,85,90,91,98]. Intranasal inoculation with high doses of some CHIKV strains can lead to potentially lethal disease in immunocompetent mice [65,87,96]. Some studies using immunocompetent mice have also examined virus loads and/or histopathological changes in other organs [66,82,104], or changes in inflammatory cytokine levels [65,96] as additional markers of infection/disease severity. Immunodeficient mice are susceptible to CHIKV challenge and provide a lethal endpoint model, although they may be less reliably informative for the relative immunogenicity of different vaccine types due to their impaired innate immune responses [63].

Cynomolgus or rhesus macaques are the primary nonhuman primate (NHP) models for CHIKV infection. Infection of these NHPs with CHIKV results in viremia (typically higher in cynomolgus), fever (most effectively measured via telemetry), rash and, when infecting with very high doses (>10E6 pfu) can be associated with joint swelling/pathology or neurological signs [109]. Changes in levels of circulating inflammatory cytokines have also been used as an additional endpoint indicative of productive infection post-challenge [105]. Recent NHP vaccination and challenge studies have typically included challenge via subcutaneous [85,110] or intravenous [101,105] routes with doses of the La Reunion 2006 OPY-1 strain ranging between 10E4-10E10 pfu (Table 3 and Supplementary Table).

Although immunogenicity and/or protective capacity of many candidate CHIKV vaccines have been assessed in small animal and NHP models, only limited assessment of minimally protective neutralizing antibody titers has occurred in those studies described in the literature. As mentioned above, the utilization of many varying assays to assess total and neutralizing antibody responses also complicates comparison of data reported from those studies. However, some general characteristics of protective neutralizing antibody responses can be inferred. In most cases, immunization leading to induction of detectable neutralizing antibodies in immunocompetent or immunodeficient mice results in robust

Table 3
Animal models for CHIKV vaccine immunogenicity and/or efficacy testing.

Animal model	Species/Strains	Immunization route(s)	Challenge route (s)/dose(s)	Clinical signs and other endpoints	Example Reference(s)
Outbred mice	CD-1, 3–8 wks	IP	IC (7000 LD50) SC (foot), (10E4 TCID50)	Mortality Viremia, footpad swelling	Levitt et al., 1986 [79]; Wang et al., 2011 [118]; Wang et al., 2011 [86]
	NIH Swiss, Swiss, Swiss Bagg; 3–5 wks old	IP SC	IC (300 LD50) None	Mortality	Eckels et al., 1970 [69]; White et al., 1972 [72]; Wang et al., 2008 [87]; Tiwari et al., 2009 [74]
	NIH Swiss, 3–5 wks old	SC	IN (10E6.5 PFU Ross strain or LR strain)	Viremia; hunching, ruffled fur, weight loss (for Ross strain)	Wang et al., 2008 [87]
Inbred mice	C57BL/6, 3–12 wks	SC, IM, IP	SC (footpad), IP (10E2-6 PFU)	Viremia, footpad swelling	Wang et al., 2011 [86]; Chattopadhyay et al., 2013 [90]; Piper et al., 2013 [82]; Metz et al., 2013a [98]; Hallengard et al., 2014a,b [83,97]; Garcia-Ariaza et al., 2014 [91]; Eldi et al., 2017 [120]; Erasmus et al., 2017 [85];
	C57BL/6; 3–6 wks	SC	IN (Ross strain, 10E6-7 pfu)	Mortality; ruffled fur, hunched posture; viremia and virus load in tissues; weight loss; slight fever	Wang et al., 2008 [87]; Plante et al., 2011 [81]; Wang et al., 2011 [86]
	BALB/c mice, 4–8 wks old	IM, SC, ID	SC (footpad or side of foot), ID (10E4-7 TCID50 or PFU)	Viremia, virus load in tissues, footpad swelling. Mortality with 10E7 PFU dose of Del-03 strain.	Akahata et al., 2010 [101]; Prow et al., 2010 [75]; Khan et al., 2012 [103]; Kumar et al., 2012; Weger-Lucarelli et al., 2015 [93]; Muthumani et al., 2016 [96]; Saraswat et al., 2016 [100]
	BALB/c mice, 3–8 wks old	IM, SC	IN (10E6-7 TCID50 or PFU)	Mortality (up to ~6 wks of age with Ross strain); viremia, virus load in tissues, weight loss	Mallilankaraman et al., 2011 [65]; Kumar et al., 2012 [66]; Tretyakova et al., 2014 [95]; Weber et al., 2015 [104]; Muthumani et al., 2016 [96]
	Hairless inbred SKH1/h mice, 12–17 wks old	Cutaneous delivery	SC (foot) (10E4 TCID50)	Viremia, foot swelling	Rudd et al., 2015 [76]
Immunodeficient mice	IFNAR; IFNAR-CD46 (for measles-vectored)	IP; (also passive transfer of serum)	ID, IP (10E2 PFU)	Mortality, signs of "severe infection", lethargy, viremia	Akahata et al., 2010 [101]; Brandler et al., 2013 [88]
	A129; 3–10 wks	SC, footpad (also passive transfer of serum via IP)	SC (footpad), ID (10E2 PFU)	Mortality, weight loss, fur ruffling, hunching, viremia, temperature (hypothermia), footpad swelling	Plante et al., 2011 [81]; Wang et al., 2011 [86]; Weger-Lucarelli et al., 2014 [93]
	AG129, 6 wks	SC, IM	IP (10E3 TCID50)	Mortality, viremia, virus load in tissues,	Metz et al., 2013b [99]; van den Doel et al., 2014 [92]
Nonhuman primate	*Macaca fasciculata* (Cynomolgus macaque)	IM, SC, ID	SC (10E5 PFU) IV (10E3-4 PFU)	Viremia, virus load in tissues, temperature (fever, hypothermia), WBC changes (lymphopenia, monocytosis), cytokine changes	Roy et al., 2014 [110]; Roques et al., 2017 [105]; Erasmus et al., 2017 [85]
	Macaca mulatta (Rhesus macaque)	IM, SC,	IM (10E5 pfu) IV (10E10 pfu)	Viremia, WBC changes (lymphopenia, neutrophilia, monocytosis)	Levitt et al., 1986 [79]; Akahata et al., 2010 [101]; Mallilankaraman et al., 2011 [65]

Abbreviations: IC – intracranial, ID – intradermal, IM – intramuscular, IN – intranasal, IP – intraperitoneal, IV – intravenous, SC – subcutaneous.

protection against challenge, with no infectious virus or viral RNA detected and no other clinical signs of infection [e.g. 76,90,98]. However, some reports have described incomplete protection despite induction of neutralizing antibodies, although this may be influenced by differences in the specific animal models employed. For example, BALB/c mice immunized with a DNA vaccine expressing CHIKV structural proteins had measurable viremia and pro-inflammatory cytokine changes following intranasal challenge with 10E7 pfu of CHIKV PC-08, despite PRNT50 titers ranging between 160 and 320 [65]. Similarly, incomplete protection was reported in CD46-IFNAR mice that had PRNT50 titers 50–450 following vaccination with a measles-vectored vaccine candidate [88], although an actual minimally protective titer was not specifically identified in that report. In the same study, passive transfer of immune sera (25uL/mouse of pooled sera with individual PRNT50 titers 1350–12150) also provided strong but incomplete protection (>80% survival) in IFNAR mice.

In NHPs, pre-challenge neutralizing antibody levels equivalent to PRNT50 >1000 appear to be associated with very robust protection against viremia, fever and/or changes in cytokines indicative of productive virus replication post-challenge [101,105,110]. In studies where PRNT50 titers ranging between approximately 80–1000 were reported for some animals, evidence of viral replication and/or mild disease has been described including low level fever [110] or changes in pro-inflammatory cytokines and/or antibody levels indicative of an anamnestic immune response [85,105]. However, use of high challenge doses of CHIKV in some NHP studies somewhat complicates the assessment of partial protection observed in these models and its relevance to protective immunity in humans following infection via mosquito bite. In at least one case [85], the contributions of cellular immunity to protection appeared to be limited although more substantial increases in CD4+ and CD8+T cells were observed following challenge in vaccinated animals compared to controls. Passive protection in immunodeficient mice utilizing doses of purified IgG derived from immunized NHPs has also confirmed the importance of antibodies in protection against CHIKV infection [101], although in that study IgG-mediated protection was not correlated with measurement of circulating levels of neutralizing antibody or identification of a minimally protective dose.

Attempts have been made to directly compare CHIKV vaccine-induced neutralizing antibody levels in NHPs or human subjects with titers in convalescent CHIK patients. Using a microneutralization assay, Mallilankaraman et al. determined that the neutralizing antibody titers in NHPs receiving a candidate CHIKV DNA vaccine (range 80–1280) were comparable to those of several convalescent human samples from India (titers 40–640) [65]. Similarly, Chang et al. reported IC50 titers using a flow cytometry-based neutralization assay ranging from approximately 4500–7500 in human vaccinees as being comparable to titers in two convalescent CHIKF patients (IC50s of 4227 and 7057) [102].

5. Potential challenges and additional considerations for definition of neutralizing antibody or other markers as correlate(s) of protection for CHIKV vaccines

In evaluating the possible definition of a CoP for CHIKV vaccines, it is useful to draw a comparison to the development and clinical evaluation of an inactivated RRV vaccine candidate [111–114]. For that vaccine candidate, studies in immunocompetent and immunodeficient mice demonstrated that levels of virus-specific total antibodies (measured by ELISA) and neutralizing antibodies (measured by a microneutralization assay), as well as protection against challenge, were vaccine dose-dependent. Passive protection studies in mice further demonstrated that protection was correlated with antibody levels, and that a stronger correlation with neutralizing antibodies over total antibodies could be determined based on serial assessment of protection with sera from vaccinees following multiple vaccine doses [112]. Comparison of vaccine-induced neutralizing antibody titers associated with both active and passive protection in animal models with those in acute phase sera from RRV patients and in sera collected approximately 20 years post-infection, suggested that neutralizing antibody titers $\geq$10 were conservatively expected to be protective [111,113,114]. Although this RRV vaccine has not been licensed, this approach provides a possible precedent for further evaluation of CHIKV vaccines and protective antibody levels to define a correlate.

Given the large number of candidate vaccines under investigation for CHIKV and the wide range of approaches taken to assess immunity and protection, the situation regarding definition of a CoP is somewhat less clear. Although ongoing CHIKV vaccine development has given additional insights into which immune response(s) might be most responsible for protection and therefore serve as potential CoPs, as described above, the comparability of these studies is a major obstacle:

1. Antigens used for vaccinations differ from inactivated cultured cells [115]; inactivated virus or tissue culture supernatants [66,69–76]; live, attenuated CHIKV strains [79–83,106]; live, chimeric vectored vaccines [86–93]; DNA vaccines [65,94,95]; virus-like particles [14,99–101] and recombinant proteins [66,103,104]. In addition, DNA vaccination with expression of a CHIKV-neutralizing monoclonal antibody has been described [96].
2. The assays and the viral isolates used to measure neutralizing antibody responses differ between labs and do not allow standardization or direct comparison of reported neutralizing activity. Although some efforts have been made in individual studies to assess comparability of standard PRNT to alternative methods, in general bridging studies are not possible because an international serological standard does not exist. However, the preparation and characterization of the "First WHO International Standard for chikungunya virus antibodies (immunoglobulin G and immunoglobulin M) (human)" was recently approved by the Expert Committee on Biological Standardization [116] which should provide a means to mitigate this gap in assessing comparability between assays.
3. The amount of CHIKV-specific IgM or IgG can be determined by ELISA. However, this approach is also not standardized and comprises either commercially available ELISAs or in-house ELISAs with different sources for CHIKV antigens. Specific relationships between levels of total antibody, neutralizing antibody and protection have not been robustly investigated in most studies reported to date.
4. A limited amount of data is available about cellular immune responses generally obtained from mice [65,83,88,88,94], and again the analytical methods are not readily comparable. However, prolonged protection requires memory B cells and is dependent on the magnitude of the innate immune response that enhances adaptive cellular responses [117]. So, further detailed analysis of innate and cellular immune responses might give rise to suitable CoP based on those responses, but the methods employed to determine antibody titers and neutralizing activity are technically less challenging and therefore are more readily standardized and validated.
5. The preclinical analyses in CHIKV vaccine development are performed in different species (mice, immune deficient mice, and non-human primates) and this also makes the comparison of potential CoPs problematic. Human data are available from three clinical trials [14,80,89,102]. However, only one study

[102] correlates the titers reached in sera of vaccine recipients with titers observed in sera of convalescent patients and thereby gives the first indications for a serological CoP.

In summary, the above mentioned points show that neutralizing antibodies correlate with protection but a serological standard has to be established to compare assays and to allow the definition of a robust serological CoP for CHIKV. Since CHIKV infections cause rapidly developing, large epidemics followed by fast decline due to the development of herd immunity, performance of large clinical efficacy trials is likely to be almost impossible, meaning that CoP (s) may be the most practical way to assess the potential efficacy of candidate vaccines efficacy and ensure licensure of a CHIKV vaccine.

Conflict of interest

None.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2018.10.033.

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