Review

Insights into Antibody-Mediated Alphavirus Immunity and Vaccine Development Landscape

Anthony Torres-Ruesta 1,2,†, Rhonda Sin-Ling Chee 1,† and Lisa F.P. Ng 1,2,3,*

Abstract: Alphaviruses are mosquito-borne pathogens distributed worldwide in tropical and temperate areas causing a wide range of symptoms ranging from inflammatory arthritis-like manifestations to the induction of encephalitis in humans. Historically, large outbreaks in susceptible populations have been recorded followed by the development of protective long-lasting antibody responses suggesting a potential advantageous role for a vaccine. Although the current understanding of alphavirus antibody-mediated immunity has been mainly gathered in natural and experimental settings of chikungunya virus (CHIKV) infection, little is known about the humoral responses triggered by other emerging alphaviruses. This knowledge is needed to improve serology-based diagnostic tests and the development of highly effective cross-protective vaccines. Here, we review the role of antibody-mediated immunity upon arthritogenic and neurotropic alphavirus infections, and the current research efforts for the development of vaccines as a tool to control future alphavirus outbreaks.

Keywords: alphavirus; antibody; immunity; alphavirus vaccine

1. Introduction

Mosquito-borne alphaviruses are Group IV viruses that belong to the family Togaviridae [1]. They are enveloped, positive-sense, single-stranded RNA viruses with a size of ≈70 nm bearing a ≈11.7 kilobases genome which encodes four non-structural proteins (nsP1, nsP2, nsP3 and nsP4) that serve as the virus’ replication machinery, and five structural proteins (capsid, E3, E2, 6K and E1) that participate in the envelope assembly process [1]. Clinically, alphavirus infections in humans result in the development of viremia followed by an onset of febrile symptoms [2]. The development of inflammatory conditions compromising joints and muscle tissues has been associated to arthritogenic alphaviruses such as chikungunya virus (CHIKV), O’nyong nyong virus (ONNV), Mayaro virus (MAYV), Ross River virus (RRV), Semliki Forest virus (SFV) and Sindbis virus (SINV) with records of persistent polyarthralgia in a fraction of patients. Conversely, neurotropic alphaviruses such as Eastern Equine Encephalitis virus (EEEV), Western Equine Encephalitis virus (WEEV) and Venezuelan Equine Encephalitis virus (EEEV) have been linked to the induction of lethal encephalitis in humans and animals [3,4].

Historically, alphaviruses have a proven record of causing massive outbreaks in susceptible populations [5–8]. Additionally, the appearance of mutations favoring their ecological fit to new vectors has fueled alphavirus propagation worldwide [9,10]. A clear example of their potential as a health threat is the re-emergence of CHIKV in 2004 after a hiatus of more than 50 years since its discovery [5]. More recently, tropical emerging alphaviruses such as ONNV and MAYV are believed to have the potential to become...
future major epidemics [11–13]. This is due, in part, to the lack of robust diagnostic tests to
differentiate alphavirus infections from other febrile tropical diseases and the absence of
continuous epidemiological surveillance masking their real potential for spread beyond
endemic areas [14–16].

Although alphavirus infections are generally not life threatening the economic and social
costs incurred during outbreaks are thought to be high [17–19]. Moreover, the lack of
approved treatments leaves management of alphavirus infections to supportive care [20].
Interestingly, a body of work suggests that the alphavirus infection triggers potent humoral
responses in exposed populations which seem to confer protection against re-infection [21].
Therefore, a better understanding of the antibody responses against alphaviruses is crucial
for the development of vaccines, which would represent a big advantage in the control of
alphavirus infections.

2. Antibody-Mediated Alphavirus Immunity
2.1. Virus-Specific Antibody Kinetics Upon Natural Infection with Alphaviruses

The current knowledge on the role of antibody-mediated immunity upon viral infec-
tion has been gathered from cohort studies following major alphavirus outbreaks. Serologi-
cal surveys following CHIKV re-emergence in 2004 reported the quick development of
IgM responses between five to seven days post-illness onset (PIO) [22,23]. IgM is generally
detectable for up to three months post-infection [24–26]. However, long-lasting IgM has
been often reported in patients with long-term CHIKV-induced polyarthritis, which
might indicate a constant antigenic stimulation due to viral persistence [27]. After
the initial detection of IgM antibodies, IgG seroconversion reportedly occurs between 4 to
10 days PIO taking over as the main immunoglobulin detected in serum [22,28]. Notably,
IgG3 antibodies become the dominant IgG subtype produced upon infection and have been
associated to efficient viral clearance and protection against chronic CHIKV symptoms [23].
Importantly, IgG responses persist for several years and might be potentially lifelong [29].

ONNV and MAYV, both closely-related to CHIKV, are re-emerging arthritogenic
alphaviruses believed to be confined to sub-Saharan Africa, and Latin America, respec-
tively [6,11,12,15]. Following the largest ONNV outbreak in Uganda involving more than
two million cases between 1959–1962 [6,30], the induction of potent neutralizing antibodies
was described [31]. The first study cohort that evaluated IgM kinetics upon ONNV infec-
tion in Uganda [32] reported the appearance of IgM antibodies during the second week PIO
which remained elevated for two months. In contrast, reports from imported ONNV cases
in Europe described detectable IgM levels as early as five days PIO [33,34]. ONNV-specific
IgG levels are increased in serum after the third week and remain high beyond two months
PIO [11,34]. However, whether IgG responses are long-lasting remains unknown. Similarly,
endemic MAYV infections are characterized by the early appearance of IgM antibodies
(3–8 days PIO) that might last for one to three months [35,36]. IgG becomes detectable
around 4–10 days PIO [35] and remains elevated after 6–12 months [37,38]. Interestingly,
unlike ONNV and CHIKV infections, persistent arthralgia has been reported in more
than half of MAYV-infected individuals and although MAYV-specific antibody responses
are critical for disease resolution it is seemingly insufficient to protect patients from the
development of chronic joint manifestations [39].

Other alphaviruses linked to continuous small outbreaks associated with arthritic
manifestations in human populations are RRV and SINV. RRV is endemic to Australia and
is responsible for approximately 4000–5000 cases annually [40]. Typically, antibody kinetics
upon RRV infection are characterized by the development of IgM titers between 7–10 days
PIO, peaking at two to three weeks and lasting for 1–3 months [41,42]. IgM response
rapidly declines after three weeks PIO as IgG becomes dominant [42,43]. Interestingly, IgM
Persistence has been reported in some RRV cohorts [41]. In one study [44], 19/116 (16.4%)
of participants had detectable IgM levels that lasted between seven months to eight years
PIO. Likewise, less prevalent SINV has also been linked to the development of persistent
virus-specific IgM levels. Although, generally, the antibody response upon SINV infection
generates IgM antibodies after 6–9 days PIO and IgG antibodies after 9–14 days, some reports described the presence of detectable IgM levels up to four years suggesting active viral replication [45–47]. The clinical relevance of persistent IgM levels following RRV and SINV infection is yet to be determined.

Neurotropic alphaviruses such as EEEV, WEEV and VEEV cause sporadic cases of human encephalitis in the Americas [4]. While the natural reservoirs for these viruses are primarily birds and equines, humans are susceptible to infection when the enzootic cycle of transmission leaks into mosquito populations with a wide range of hosts [48]. Given that human cases are rare, there is a lack of information regarding the development of antibody responses upon natural infections by neurotropic alphaviruses. In a paired serology study [49], virus-specific antibody responses were profiled in a cohort of 20 EEEV and 17 WEEV-infected patients. IgM antibodies were observed as early as 1 PIO, peaking after 1–2 weeks and remaining detectable for up to three months. In contrast, IgG responses appeared during the second week PIO and remained elevated until the end of the follow-up period.

2.2. Experimental Evidence of the Role of Antibodies in Alphavirus Immunity

To better understand the role of antibody-mediated immunity upon alphavirus infection, several animal models have been used allowing the detailed examination of the cellular compartments responsible for the initiation of humoral immunity. The role of B cells in alphavirus immunity has been described in experimental CHIKV infections. Inoculation of µMT mice (lacking mature B cells) with CHIKV resulted in higher viremia that persisted up to 402 days post-infection (DPI). In contrast, infected wild type (WT) mice were able to control the virus during the second week post-inoculation [50]. Similar findings were reported in other studies, where mouse strains lacking B cells (µMT, Rag1, Rag2/IL2rg, NRG) infected with CHIKV displayed increased and persistent viremia for up to 515 DPI [51,52].

B cells also play an important role in alphavirus-induced encephalitis. Although SINV infections in humans are known to cause arthritic manifestations, SINV has been frequently used as a model of alphavirus-induced encephalomyelitis in adult immunocompetent mice given the virus ability to infect neurons [53]. Intracerebral inoculation of SINV in µMT and severe combined immunodeficiency (SCID) mice resulted in defective viral clearance from the brain, brain stem and lumbar spinal cord, virus persistence and recrudescence compared to WT mice [54]. The individual contributions of IgM and IgG antibodies to SINV clearance from brain tissues were assessed in another study [55] where infection in AID−/− (unable to produce IgG), slgM−/− (unable to produce IgM) and AID−/− slgM−/− double-knockout mice resulted only in AID−/− slgM−/− being unable to control infection efficiently suggesting that either IgM or IgG antibodies are sufficient to clear SINV from the central nervous system (CNS). Similar results were obtained in SFV models of encephalitis where infection of µMT [56], SCID [57] and nude mice with impaired antibody switching [58] led to viral persistence.

Infiltrating virus-specific B cells were observed in infected tissues in a murine model of SINV-induced encephalitis [59,60]. Following intracranial virus inoculation, expansion of IgM-secreting plasmablasts was reported in the cervical lymph nodes. Infiltration of CD19+ B cells occurred between 3–7 DPI and coincided with the starting of viral clearance. During the clearance of persistent viral RNA (from 8–80 DPI), the accumulation of SINV-specific IgG and IgA-secreting B cells was observed being associated with increased SINV antibody titers over time [60]. In a subsequent study, it was reported that the brain microenvironment during the early stages of SINV infection facilitates the migration, differentiation, expansion and long term survival of SINV-specific B cells [59].

Follicular helper T cells (TFH) are a subset of CD4 T cells involved in the activation of B lymphocytes and the establishment of robust antibody responses following antigen stimulation. TFH promotes B cell differentiation, isotype switching and affinity maturation. In experimental CHIKV infections, the use of CD4-deficient mice ruled out the role of
CD4 T cells in viral clearance from infected tissues [61]. However, one study demonstrated impaired IgM and IgG (IgG2c, IgG1, and IgG2b) production in mice lacking CD4 T cells following CHIKV inoculation [62]. Albeit reduced virus-specific antibody levels, the neutralizing capacity of sera from virus-infected CD4-deficient mice was marginally affected [62]. Likewise, another study showed similar results upon CHIKV inoculation of MHCIIΔ/Δ mice (defective of Tfh) [51]. MHCIIΔ/Δ animals were unable to generate IgG1 antibodies and produced ≈100 fold lower IgG2c levels than WT controls. Nonetheless, MHCIIΔ/Δ mice were still able to control virus infection [51]. The generation of virus-specific neutralizing antibodies in MHCIIΔ/Δ mice suggests a T-cell independent B cell activation characterized by the inability to generate memory B cells. Whether CHIKV-specific antibody responses in mice lacking CD4 T cells are long-lasting remains to be elucidated.

2.3. Viral Antigenic Regions Targeted by Neutralizing Antibodies

The notion of targeting humoral immunity as a therapy against alphavirus infection has been investigated since the late 1930s following the isolation of EEEV, WEEV and VEEV. In a series of seminal studies involving immunization of guinea pigs [63–66], the subcutaneous inoculation of live EEEV and WEEV strains protected guinea pigs from lethal intracranial infection [63]. Additionally, it was observed that immunization with formalin-inactivated virus strains induced the production of neutralizing antibodies at a comparable level than animals immunized with live viruses [64–66]. Subsequent studies reported that passive transfer of hyperimmune rabbit serum protected mice, guinea pigs and rabbits from WEEV infection [66,67]. Similarly, passive serum transfer was shown to be effective at protecting mice from the development of neurological complications upon infection with a neuroadapted strain of SINV [68,69]. Comparable observations were reported in experimental infection models of VEEV [70], CHIKV [71,72], RRV [73] and SFV [74].

The first attempts in identifying the exact structural regions, recognized by most neutralizing antibodies produced upon infection, were conducted in experimental infection models of alphavirus encephalitis. Structurally, the envelope of an alphavirus virion has a T = 4 icosahedral symmetry [75]. E1 and E2 are two envelop surface glycoproteins exposed in the viral spike as a heterodimer [75] (Figure 1). It is believed that the E1-E2 heterodimer interacts with host receptors thus mediating viral entry [75]. Additionally, the E1 and E2 glycoproteins were postulated as highly immunogenic regions since their location in the spike facilitates antigenic recognition. In line with this, early works mapped antigenic sites involved in VEEV, SINV and SFV neutralization to the E1 and E2 proteins using competitive binding assays but the exact amino acid sequences were not determined [76–78]. Later, a major antigenic region involving three epitopes important in the neutralization of RRV was identified in the E2 protein (incorporating residues 216, 232 and 234) [79]. Similarly, analysis of antibody escape variants determined important antigenic regions between amino acids 181 and 216 on the E2 protein of SINV [80]. A major neutralization domain was also identified between residues 182–207 for VEEV [81].

Following CHIKV reemergence in 2004 several reports identified major linear antigenic sites in the CHIKV E2 protein that induced the production of potent neutralizing antibodies. Using a CHIKV proteome-wide screening approach, a single linear peptide located at the N-terminus of the E2 glycoprotein, E2EP3, was reported as strongly recognized by convalescent CHIKV patients from different cohorts [23]. Furthermore, experimental CHIKV infection in mice and non-human primates (NHP) validated E2EP3 as an immunodominant linear epitope inducing potent neutralizing antibodies [23,62,82]. Interestingly, mice immunization with E2EP3 alone reduced joint swelling and viremia upon CHIKV challenge [23]. In another study focusing on human antibody responses to SINV in cohort from Finland, 6 linear epitopes, located in the capsid, E2, E1 and PE2 (uncleaved E3-E2) proteins, were reported [83]. Three of these epitopes were located to the glycoprotein spike complex between the residues 209–226 of E1 (E1-P5), 273–290 (E2-P3) and 308–325 (E2-P4)
of E2 [83]. Interestingly, the E2EP3 equivalent of SINV remained non-reactive suggesting that antibody kinetics against linear E2EP3 between populations exposed to CHIKV and SINV might differ [83].

**Figure 1.** Structure of the alphavirus E1-E2 heterodimer. Ribbon diagram (PDB: 3N41) highlighting (A) E1 glycoprotein (domain I: red, domain II: yellow, domain III: blue, fusion loop FL: orange, E2: grey) and (B) E2 glycoprotein (domain A: cyan, domain B: green, domain C: pink, beta-ribbons: purple, E1: grey). (C) Table summarizing reported antibody binding regions in the E1 and E2 glycoproteins of arthrogenic and neurotropic alphaviruses. Numbers in the table refer to in-text citations describing such binding sites (See Reference list). Background color matches protein doimains depicted in (A) and (B). To assess for the degree of conservation among common antigenic regions across alphaviruses a sequence alignment analysis was conducted (See Supplementary Figure S1).
The development of mouse and human monoclonal antibodies against different alphaviruses helped further the understanding of antigenic responses upon infection by the identification of conformational epitopes. Early works have shown the therapeutic value of mouse monoclonal antibodies in models of alphavirus encephalitis by SINV [84–88], SFV [56,57,89] and VEEV [78]. Interestingly, it was observed that neutralizing monoclonal antibodies target antigenic regions in the E2 protein. Whereas, non-neutralizing antibodies bind to the E1 protein, yet both are able to confer protection upon alphavirus infection, thereby suggesting other mechanisms of protection in vivo besides virus neutralization [48]. Several monoclonal antibodies targeting both E1 and E2 proteins have been reported in the context of arthritogenic alphavirus infection. Mouse monoclonal antibodies targeting the A and B domain of E2 and the domain II of E1 [90–92] and the capsid protein [93,94] have been reported for CHIKV. Likewise, human anti-CHIKV monoclonal antibodies were found to target conformation epitopes in the E2 glycoprotein A (containing a putative RBD [95]) and B (shielding the fusion loop in E1 [96]) domains and proved therapeutic value in experimental NHP infections [90,97,98]. Monoclonal antibodies recognizing epitopes predominantly between residues 58–80 (domains A) or residues 180–215 (domain B) of the E2 glycoprotein have been also reported in the context of SINV [83], VEEV [81], EEEV [99–101], RRV [102] and MAYV [103].

The combined evidence suggested the existence of common antigenic sites in the viral spike across alphaviruses, particularly in the E2 protein. These sites are likely required for interaction with host cell receptors suggesting that antibody binding might inhibit infection during viral attachment, entry, fusion or egress [90]. In line with this, a recent study reported the discovery of Mxra8, a cell adhesion molecule, as a host receptor required for viral entry of multiple arthritogenic alphaviruses [104]. Genetically altering mouse or human Mxra8 resulted in diminished infection, conversely, overexpression of Mxra8 in cell lines increased infection rates by CHIKV, ONNV, MAYV and RRV [104,105]. Interestingly, mutagenesis experiments suggested E2 domains A and B as the putative binding site for Mxra8 [104]. This notion was later confirmed by cryo-electron microscopy images of Mxra8 bound to CHIKV [106,107]. Mxra8 sits onto a cleft formed by two contiguous CHIKV E2-E1 heterodimers in one trimeric spike while engaging a neighboring spike [106]. It is believed that this interaction works against the virus by obstructing viral fusion [106]. Importantly, human neutralizing antibodies that recognize regions of the A domain of E2 inhibited the binding of Mxra8 supporting the interactions determined in the cryo-EM atomic model. Notably, Mxra8 seems to not be a receptor for neurotropic alphaviruses [104]. The alignment of CHIKV residues involved in Mxra8 binding revealed a degree of conservation in arthritogenic alphaviruses (44%), but diverged from neurotropic Alphaviruses (14%) which might explain the negative results in the context of SINV, EEEV, WEEV and VEEV infections [106]. In summary, the characterization of alphavirus antigenic epitopes has proven beneficial to pave the way for the development of antibody therapies and vaccines.

3. Alphavirus Vaccine Development

Recent decades have seen increased rates of geographic dispersal of arboviral re-emergence, due to factors such as growth of global transportation, urbanization and failure of mosquito control [108–111]. Given that humans appear to be the only amplification hosts and viral reservoir during urban transmission [112,113], another effective means of controlling the spread of infection is through vaccination. While there are currently no licensed or approved vaccines available for alphaviruses, a multitude of approaches have been used to develop vaccine candidates capable of, not only generating high levels of antibodies, but also providing long-lasting protection, with the ease of administration and production requirements. Multiple methods such as live-attenuated viruses, inactivated viruses, virus-like particles (VLP), recombinant subunit vaccines and chimeric vaccines have been explored for vaccine options (Figure 2 and Table 1).
Figure 2. An outline of the current vaccine options against arthritogenic (left panel) and neurotropic (right panel) alphaviruses. Most of these vaccine candidates are currently under preclinical testing (early preclinical—vaccine candidates tested in mouse models; late preclinical—vaccine candidates currently under testing in non-human primates (NHP)), while a minority of them are currently undergoing clinical trials (Phase 1, 2 or 3). LAV; live-attenuated virus; VLP, virus-like particle; SIN, Sindbis virus; ISFV, Isfahan virus; May, Mayaro virus; EILV, Eilat virus, VSV/VSIV, vesicular stomatitis virus; MV, measles virus; MVA, modified vaccinia virus Ankara. Data curated from literature reported through February 2021. Numbers in superscript refer to reference numbers (See Reference list [23,73,114–220]).
| Vaccine Against Virus | Name | Strain Vaccine Modeled After | Phase | Immunization | Challenge | Humoral Immune Response(s) | Ref |
|-----------------------|------|-----------------------------|-------|--------------|-----------|---------------------------|-----|
|                       |      |                             |       |              | Dose (Strain, Genotype)   | Route |                           |     |
|                       |      |                             |       |              | Dose | Route | Schedule |              |     |
| Live-attenuated       |      |                             |       |              |      |      |          |              |     |
| CHIKV, ONNV           | RH-CHIKV | LR2006 OPY1 | C57BL/6 mice, 3 week old | 10^6 PFU | s.c. in the ventral side of the right hind footpad | Single dose | 10^6 PFU LR2006 OPY1 or WT-ONNV IMTSSA/5163, 3 mpim | s.c. in the ventral side of the right hind footpad | IC50, 613 (RH-CHIKV), 3407 (EV-CHIKV), 921 (RHEV-CHIKV) | [130] |
|                       | EV-CHIKV | LR2006 OPY1 | C57BL/6 mice, 3 week old | 10^6 PFU | s.c. in the ventral side of the right hind footpad | Single dose | 10^6 PFU LR2006 OPY1, 7 wpim | s.c. | NT50, 100 to 1000 |     |
|                       | RHEV-CHIKV | LR2006 OPY1 | C57BL/6 mice, 3 week old | 10^4 or 10^5 PFU | s.c. in both flanks | Single dose | 100 AID50 (corresponding to 7000–10,000 PFU) LR2006 OPY1, 123 dpim | i.v. | NT50, >1000 | [131–133] |
|                       | (VLA1553-301 in clinical trials) and Δ6K | LR2006 OPY1 | Cynomolgus macaques, 3–4 years old | 10^5 PFU | s.c. in both flanks | Single dose | 10^6 PFU LR2006 OPY1, 7 wpim | s.c. | NT50, 100 to 1000 |     |
|                       |                  |                              | Human clinical trial, Phase 1 | 3.2 × 10^3, 3.2 × 10^4 or 3.2 × 10^5 TCID50 | i.m. | Two doses (0 and 6 months, or 0 and 12 months) | NA | NA | GMT, 592.6 to 686.9 |
| CHIKV                 | CHIKV-NoLS | LR2006 OPY1 | C57BL/6 mice, 21 days of age | 10^4 PFU | s.c. | Single dose | 10^4 PFU of LR2006 OPY1 or Ross River virus, 30 dpim | s.c. | <10% cells infected at 10^3 serum dilution | [127] |
| CHIKV                 | Stop CHIKV | LR2006 OPY1 | C57BL/6 mice, 5 week old | 10^4 PFU | s.c. | Single dose | ND | ND | ~5–25 (Stop CHIKV) and ~10–25 (SuperStop CHIKV) fold reduction compared to mock | [134] |
| CHIKV                 | SuperStop CHIKV | LR2006 OPY1 | C57BL/6 mice, 5 week old | 10^4 PFU | s.c. | Single dose | ND | ND | ~5–25 (Stop CHIKV) and ~10–25 (SuperStop CHIKV) fold reduction compared to mock | [134] |
| CHIKV                 | ChikV HR | 37997 | C57BL/6 mice, 28 days of age | ~10^3 PFU | s.c. into the left footpad | Single dose | 10^3 PFU CHIKV SL15649, 28 dpim | s.c. in the footpad | PRNT50, 5 to ~500 | [135] |
| CHIKV                 | Heparin sulfate cell culture adapted | LR2006 OPY1 | CD-1 mice, 21 days old | 10^5 GE | s.c. in the rear footpad | Single dose | 10^3 PFU LR2006 OPY1, 21 dpim | NA | ~40 to 1000 fold change compared to mock | [136] |
| Vaccine Against Virus | Name | Strain Vaccine Modelled After | Phase | Immunization | Challenge | Humoral Immune Response(s) | Ref |
|-----------------------|------|------------------------------|-------|--------------|-----------|---------------------------|-----|
| VEEV                  | V326 | IA/B Trinidad donkey         | BALB/c, 6 to 8 week old C3H/HeN mice, 6 to 8 week old | 10⁵ PFU | s.c. | Single dose | 10⁵ PFU of TrD, 28 dpim | NP | ND |
|                       |      |                              | Cynomolgus macaques (age not specified) | 2.5 × 10⁶ PFU | s.c. | Single dose | ~10⁶ PFU VEEV IE 68U201, 8 wpim aerosol | PRNT80, 28 to 2560 | [137–140] |
|                       |      |                              | Rhesus macaques (2 to 4 years old) | 1.3 × 10⁵ or 7.5 × 10⁴ PFU | s.c. or i.t./i.s. | Single dose | ND | ND |
|                       |      |                              | Human clinical trial, Phase 1 | 25 or 125 PFU | s.c. | Single dose | NA | NA |
|                       |      |                              | BALB/c mice, 4 to 8 week old | 10⁴ PFU | s.c. | Single dose | 10⁴ PFU of VEEV TrD, 28 dpim | s.c. | PRNT80, 160 to1280 | [141,142] |
|                       | V420 | IA/B Trinidad donkey         | Cynomolgus macaques (age not specified) | ~10⁴ PFU | s.c. in the right leg | Single dose (or second dose at 2 x 10⁴ PFU i.m. if did not seroconvert) | 10⁶ to 10⁷ PFU of the VEEV TrD, 73 dpim aerosol | PRNT80, >640 |
| EEEV                  | FL93-939 | 5′U4&6 C65-69 E71-77 3′U11337 mutants | CD-1 mice, 5 to 6 week old | 1.5 × 10⁵ GE | s.c. in footpad, or i.c. | Single dose | 10⁵ PFU EEEV FL93, 21 dpim | s.c. in both footpads | PRNT80, 16 to ~4000 | [143] |
| CHIKV                 | CHIKV/RES LR2006 OPY1 |                               | A129 mice, 3 or 10 week old | 10⁴ PFU | i.d. | Single dose | 100 PFU LR2006 OPY1, 94 dpim | i.d. | PRNT80, >320 |
|                       |      |                               | C57BL/6 mice, 3 week old | 10⁵ PFU | s.c. in the hind leg | Single dose | 10⁵.5 PFU Ross CHIKV, 21 dpim | i.n. | Mean PRNT80, 62 |
|                       |      |                               | A129 mice, 8 to 10 week old | 10⁵ TCID50 | s.c. | Single dose | 100 PFU LR2006 OPY1, 50 dpim | i.d. | Mean PRNT80, 1152 |
|                       |      |                               | Cynomolgus macaques, >3 years old | 10⁵ PFU | s.c. or i.d. | Single dose | 10⁵ PFU LR2006 OPY1, 52 dpim | s.c. in the upper deltoid | PRNT80, 40 to 640PRNT50, 160 to 1280 | [144,145] |
| Vaccine Against Virus | Name | Strain Vaccine Modelled After | Phase | Immunization | Challenge | Humoral Immune Response(s) | Ref |
|-----------------------|------|-----------------------------|-------|--------------|-----------|---------------------------|-----|
| **ONNV** | CHIKV/IRES | LR2006 OPY1 | A129 mice, 6 to 7 week old | 10⁴ PFU | i.d. | Single dose | 10⁵ PFU ONNV SG650, 38 dpim | i.d. | PRNT80, 160 | [146] |
| **VEEV** | ZPC/IRESv1, ZPC/IRESv2 | ID ZPC738 | CD-1 mice, 6 to 8 week old | 10⁵ PFU | s.c. in the scruff of the back | Single dose | 10⁵ PFU VEEV 3908, 4 wpim | s.c. or aerosol | PRNT80, 40 to 232 | [114] |
| | | | Cynomologous macaques, age not specified | 10⁵ PFU | s.c. in the upper deltoid | Single dose | ~ 8 × 10⁷ to 9 × 10⁸ PFU VEEV 3908, 35 dpim | aerosol | PRNT80, <20 to 20/PRNT50, <20 to 160 |
| **EEEV** | EEE/IRES | FL93-939 | NIH Swiss mice, 3 to 4 week old | 10⁴ PFU | s.c. in the medial thigh | Single dose | 10³ PFU of FL93-939, 4 wpim | i.p. | PRNT80, 160 to 640 | [147] |
| **VEEV** | 68U201/ IRESv1, 68U201/ IRESv2 | IE 68U201 | CD1 mice, 6 to 8 week old | 10⁵ PFU | s.c. in right hind leg | Single dose | (Lethal dose, NP) 68U201 at 1, 3, or 12 mpim | s.c. | PRNT80, 64 to ~300 | [148,149] |
| | | | Cynomologous macaques (age not specified) | 10⁵ PFU | s.c. in the upper deltoid | Single dose | 4 × 10⁴ PFU VEEV IE 68U201, 49 dpim | Aerosol | PRNT80, −100 to 340 |
| **VEEV** | VEEV/IRES/C | IA/B Trinidad donkey | CD-1 mice, 8 week old | 10⁴ PFU | s.c. | Single dose | 10⁴ PFU of VEEV 3908, 6 wpim | s.c. | Mean PRNT80, 184 | [150] |
| **MAYV** | MAYV/IRES | MAYV-CH | BALB/c, 6 week old | 2 × 10⁵ PFU | s.c. i.pl. route | Single dose | 2 × 10⁵ PFU of WT MAYV, 28 dpim | s.c. i.pl. route | PRNT50, >640 (at 21dpi) | [146,151, 152] |
| | | | AG129 | 2 × 10⁴, 2 × 10³ or 2 × 10² PFU | s.c. i.pl. route | Single dose | 2 × 10⁵ PFU of WT MAYV, 14 dpim | s.c. i.pl. route | ND |
| | | | CD-1, 28-day old | 10⁵ PFU | s.c. over the dorsum | Single dose | ND | ND | PRNT80, 160 to >640 |
| | | | AG129, 5 to 8 week old | 10⁴ PFU | i.d. on the left foot | Single dose | 10⁴ PFU of WT MAYV, 29 dpim | s.c. | PRNT80, 320 to >640 | |
| **Inactivated** | **CHIKV** | Vero cell adapted | DRDE-06 | Swiss albino mice, 3 to 4 week old | 10, 25 or 50 ug | s.c. | Three doses (0, 14 and 28 days) | ND | ND | PRNT90, 6400 | [153] |
| Vaccine Against Virus | Name | Strain Vaccine Modelled After | Phase | Immunization | Challenge | Humoral Immune Response(s) | Ref |
|-----------------------|------|------------------------------|-------|--------------|-----------|-----------------------------|-----|
| CHIKV                | BPL/formalin- | CHIKV BBV87 (in clinical trials) | BALB/c mice, 4 to 6 week old | 10, 20 or 50 µg | i.m. | Two doses (0 and 14 days) | 2.5 x 10^4 TCID50 IND-06-AP3, 4 or 22 wpim | i.n. | GMT, NT50, 80 to 1280 | [154] |
|                      | inactivated |                             | Human clinical trial, Phase 1 | 10, 20 or 30 µg | i.m. | Three doses (0, 29 and 57 days) | NA | NA | NA | [155] |
| RRV                  | Vero cell culture-derived whole-virus RRV vaccine Ross River Virus (RRV) Vaccine | T48 | CD-1 mice, 7 to 8 week old | 0.0025, 0.01, 0.039, 0.156, 0.25, or 10 µg | s.c. | Two doses (0 and 28 days) | 10^6 TCID50 RRV T48, 42 dpim | i.v. | Mean NT, ≤2.9 to 46.2 | [73,115, 128,129] |
|                      |                             | A129 mice, 7 to 8 week old | 0.063, 0.25 or 1 µg | i.m. | Two doses (0 and 21 days) | 10^2.5 TCID50 T48, 42 dpim | s.c. into left footpad | Mean NT, ≤14 to 21 | |
|                      |                             | CD-1 mice, age not specified | 10 µg | s.c. | Two doses (0 and 28 days) | 10^6 TCID50 T48, 6 wpim | i.v. | 1000 TCID50 | |
|                      |                             | Guinea pigs (Duncan Hartley), age not specified | 10 µg | s.c. | Single or two doses (0 and 6 weeks) | 10^6 TCID50 T48, 10 or 34 wpim | i.v. | NP | |
|                      |                             | Human clinical trial, Phase 1/2 | 1.25, 2.5, 5, or 10 µg | i.m. | Three doses in escalation (0, 21 days, 6 months) | NA | NA | GMT, 50 to 520.9 | |
|                      |                             | Human clinical trial, Phase 3 | 2.5 µg | i.m. | Three doses (0, 3 weeks, 6 months) | NA | NA | µNT GMT, −0 to 85 | |
| EEEV                 | TSI-GSD-104 (formalin inactivated) | PE-6 | Human clinical trial, Phase 2 | s.c. (0 and 28 days), i.d. (6 months) | Three doses (0, 28 days and 6 months) | NA | NA | PRNT80 >40 in 60% subjects (primary doses) versus 84% subjects (completed the 2-dose primary series and the 6-month dose) | [156–158] |
| Vaccine Against Virus | Name | Strain Vaccine Modelled After | Phase | Immunization | Challenge | Humoral Immune Response(s) | Ref |
|-----------------------|------|------------------------------|-------|--------------|-----------|--------------------------|-----|
| EEEV | fCVEV1219/ gCVEV1219 | CVEV1219 | BALB/c mice, 6 to 8 week old | 0.1 to 5 µg of inactivated EEEV | Single dose or two doses (0 and 28 days) | Lethal dose of EEEV FL93-939, at 28 dpim (single dose) or 56 dpim (two doses) | aerosol | PRNT80, –1 to 1000 |
| VEEV | V3526 virus | V3526 | BALB/c mice, 6 week old | 0.2 µg (s.c.) or 0.04 µg (i.m.) | Two doses (0 and 28 days) | 10⁴ PFU VEEV TrD, 56 dpim | aerosol or s.c. | GMT PRNT80, –60 to 2500 |
| VEEV | F-Iv3526 | V3526 | BALB/c mice, 8 to 10 weeks old | 1, 3 or 5 µg | i.n., s.c. (under the skin over the neck) or i.m. (thigh muscle of the hind leg) | Single dose | 454 (i.n.), 897 (i.m.) or 55 (s.c.) PFU VEEV-TrD, 56 dpim | aerosol | Microneutralization titer of 100 to 3500 |

**Virus-like particle**

| Vaccine Against Virus | Name | Strain Vaccine Modelled After | Phase | Immunization | Challenge | Humoral Immune Response(s) | Ref |
|-----------------------|------|------------------------------|-------|--------------|-----------|--------------------------|-----|
| CHIKV | VRC 311/ Or VRC-CHKVLP09-00-VP/ PVXV0317 (in clinical trials) | 37997 | BALB/c mice, 6 to 8 week old | 19 µg | i.m. | 2 doses (2 and 5 weeks) | ND | ND | IC50, 10,703 to 54,600 |
| | | | Cynomolgus macaques, 3 to 4 years old | 20 µg | i.m. | 3 doses (0, 4 and 24 weeks) | 10¹⁰ PFU LR2006 OPY1, 15 wpim | i.v. | IC50, 10,219 to 15,072 |
| CHIKV | Baculovirus-expressed VLP | S27 | AG129, 6 week old | 1 µg | s.c. | 2 doses (0 and 21 days) | 1000 TCID50 S27, 6 wpim | i.p. | PRNT95, 40 to 80 |
| | | | C57BL/6 mice, 6 to 12 week old | 0.1 or 1 µg | s.c. | Single dose | 10⁴ CCID50 LR2006 OPY1, 6 wpim | s.c. | NT95, –1,100 |
| CHIKV | Yeast-expressed VLP | DRDE06/DRDE07 | BALB/c mice, 4 week or 2 days old | 10, 20 or 40 µg | s.c. | Three doses (0, 14 and 28 days) | ND | ND | NT50, 128 to 2048 |
Table 1. Cont.

| Vaccine Against Virus | Name | Strain Vaccine Modelled After | Phase | Immunization | Challenge | Humoral Immune Response(s) | Ref |
|-----------------------|------|-----------------------------|-------|--------------|-----------|---------------------------|-----|
| VEEV                  |      | NA                          | Human clinical trial (Phase 1, not recruiting) | 2, 10, or 20 µg i.m. | Dose escalation (0, 28 days, and day 140 booster) | NA | NA | NA | [167] |
| WEEV, EEEV, and VEEV | VRC-VEVVLP073-00-VP (Trivalent vaccine) | BALB/c mice, 6 to 8 week old | monovalent (5 µg) or trivalent (5 µg each) i.m. | Two doses (0 and 21 days) | 2.5 × 10^3 PFU WEEV CBA87, 8.9 × 10^3 PFU EEEV FL93-939, and 1.3 × 10^3 PFU VEEV Trinidad donkey, 56 dpim aerosol PRNT80, −250 to 100000 | [168] |
| DNA/RNA               |      |                             | Cynomolgus macaques, age not specified | Monovalent (20 µg) or trivalent (20 µg each) i.m. | Two doses (0 and 28 days) | 10^6 PFU WEEV CBA87, 10^8 PFU EEEV FL93-939, and 10^8 VEEV Trinidad donkey, 56 dpim aerosol PRNT80, −1000 to 10000 | [169] |
| VEEV                  | VEEV 26S DNA plasmid 1/AB TrD | BALB/c mice, 6 to 8 week old | ~3 µg DNA/gene gun, delivered to two sites on the abdomen of each mouse | Three doses (at 3-week intervals) | ~10^4 PFU of TrD, 9 wpim s.c., aerosol PRNT50, GMT <1.6 to 2.5 | [170,171] |
|                       |      | Hartley guinea pigs, age not specified | ~5 µg DNA/gene gun, delivered to two sites on the abdomen of each mouse | Three doses (0, 4 and 8 weeks) | ~10^4 PFU of TrD, 21 wpim aerosol PRNT50, 0 to 640 | |
### Table 1. Cont.

| Vaccine Against Virus | Name          | Strain Vaccine Modelled After | Phase                     | Immunization                                             | Challenge                                                                 | Humoral Immune Response(s) | Ref |
|-----------------------|---------------|------------------------------|---------------------------|----------------------------------------------------------|---------------------------------------------------------------------------|-----------------------------|-----|
| VEEV                  | DNA-Ad        | TC-83 BALB/c mice, 6 to 8 week old | 1 µg of DNA per dose and 107 PFU of RA02/VEEV #3 per boost | gene gun.i.n. \[immunised with the DNA vaccines on day 0, 14 and 28 and Ad-based vaccine on day 42\] | 100 LD50 of virulent airborne VEEV, 63 dpim | aerosol | PRNT50, 160 | [172] |
| VEEV                  | AG4-1C7 AG4-1G2 AG2-5A7 AG2-5A10 plasmid DNA | 1/ AB TrD BALB/c mice, 6 to 8 week old | 4 µg particle-mediated epidermal delivery (i.d.) | Three doses (at 3-week intervals) \[\sim10^4 PFU of VEEV TrD (\geq1000 LD50), 70 dpim\] | aerosol | PRNT80, –1 to 5.5 log_{10} GMT | [173] |
| VEEV                  | pTC83 iDNA    | TC-83 BALB/c mice, 3 week old | 50 µg i.m. electroporation | Single dose \[10^5 PFU VEEV 3908, 21 dpim\] | s.c. | PRNT80, 10 to 320 | [174] |
| WEEV                  | pE3-E2-6K-E1  | 71V-1658 BALB/c, age not specified | 2 µg gene gun | Three doses (14 days apart) \[\sim10^4 PFU WEEV 71V-1658, Fleming, or CBA87, 42 dpim\] | i.n. | ND | [175] |
| CHIKV                 | pCHIKV-Capsid, pCHIKV-Envelope (pMCE321) | Consensus C57BL/6 mice, 3 to 4 week old | 25 µg, 2–3 times Electroporation | Two doses (2 weeks apart) \[ND\] | ND | ND | \[176–178\] |
| CHIKV                 | pCHIKV-Capsid, pCHIKV-Envelope (pMCE321) | Consensus C57BL/6 mice, 6 to 8 week old | 25 µg i.m. electroporation | Three doses (0, 14 and 21 days) \[\sim10^5 PFU of PC-08, 35 dpim\] | i.n. | NP | \[176–178\] |
| CHIKV                 | BALB/c mice   | pCHIKV-Capsid, pCHIKV-Envelope (pMCE321) | 25 µg i.m. electroporation | Two doses (2 weeks apart) \[\sim10^5 PFU PC-08\] | i.n. | TCID50, 20 to 320 | \[176–178\] |
| CHIKV                 | BALB/c mice   | pCHIKV-Capsid, pCHIKV-Envelope (pMCE321) | 1 mg i.m. electroporation | Three doses (4 weeks apart) \[ND\] | ND | TCID50, 80 to 1280 | \[176–178\] |
| CHIKV                 | Δ5nsP3 and Δ6K DNA | LR2006 OPY1 C57BL/6 mice, 5 to 6 week old | 20 µg i.d. with DermaVax electroporation | Single dose or two doses (0 and 3 weeks) \[\sim10^5 PFU LR2006 OPY1, 7 wpim\] | s.c. | NT50, 100 to 10000 | [131] |
| Vaccine Against Virus | Name | Strain Vaccine Modelled After | Phase | Immunization | Challenge | Humoral Immune Response(s) | Ref |
|-----------------------|------|-----------------------------|-------|--------------|-----------|---------------------------|-----|
| CHIKV                | CHIKV-NoLS RNA | LR2006 OPY1 | C57BL/6 mice, 28 days of age | 2 µg (s.c.) in the ventral/lateral side of the right foot | Single dose 10⁴ PFU LR2006 OPY1, 30 dpim | s.c. in the ventral/lateral side of the right (ipsilateral) or left (contralateral) | PRNT80, 0 | [126] |
|                      |       | AG129 mice, 28 days old     | 2 µg (s.c.) in the ventral/lateral side of the right foot | Single dose 10⁴ PFU LR2006 OPY1, 30 dpim | s.c. in the ventral/lateral side of the right (ipsilateral) or left (contralateral) | ND | |
| VEEV, WEEV and EEEV  | 3-EEV | VEEV IAB TrD, WEEV CBA874 and EEEV FL91-46794 | C57BL/6 mice, 6 to 8 week old | 15 µg (i.m. electroporation) | Two doses (0 and 21 days) 10⁴ PFU VEEV IAB TrD or 2 × 10⁴ PFU WEEV CBA874 or 10⁵ PFU EEEV FL91-46794, 7 wpim | aerosol | PRNT80, ~1 to 1000 | [179] |
| MAYV                 | scMAYV-E | NA | C57BL/6 mice, 5 to 8 week old | 25 µg (i.m. electroporation) | Single, two doses or three doses (at 2 week intervals) ND | ND | PRNT50, 789.8 | [180] |
|                      |       | A129 mice, 4 to 6 week old  | 25 µg (i.m. electroporation) | Single, two doses or three doses (at 2 week intervals) | 10² PFU MAYV 15537 | i.p. | ND | |
| CHIKV                | p181/25-7 | TSI-GSD-28 | BALB/c mice, 3 week old | 10 µg (i.m. electroporation) | Single dose 6 × 10⁶ PFU CHIKV Ross, 28 dpim | i.n. | PRNT80, 160 to 1280 | [181] |
| CHIKV                | dMaB  | NA | BALB/c mice, age not specified | 100 µg (Electroporation) | Single dose 10⁷ PFU Del-03 | s.c. or i.n. | ICS0, 3 to 4.5log₁₀ | [182] |
Table 1. Cont.

| Vaccine Against Virus | Name | Strain Vaccine Modelled After | Phase | Immunization | Challenge | Humoral Immune Response(s) | Ref |
|-----------------------|------|------------------------------|-------|--------------|-----------|---------------------------|-----|
| CHIKV                | iRNA∆5nsP3 iDNA∆5nsP3 | LR2006 OPY1 | C57BL/6 mice, 8 week old | 0.125, 1.25 or 10 µg i.m. in the gastrocnemius muscle of the left hind leg | Single dose | 10^8 PFU LR2006 OPY1, 5 wpim | s.c. at the dorsal side of each hind foot | NT50, ~1 to 10^4 | [183] |
| VEEV                 | pMG4020 DNA plasmid | TC-83 | BALB/c, 4 to 8 week old | 0.5 or 5 µg i.m. electroporation | Single dose | 10^4 PFU VEEV TrD, 28 dpim | s.c. | PRNT80, 320 to >1280 | [141] |
| VEEV                 | VEEV<sub>NT</sub> VEEV<sub>COCAP</sub> VEEV<sub>CO</sub> | IAB TrD | BALB/c, 6 to 8 week old | 25, 5, or 1 µg i.m. electroporation | Two doses (3 weeks apart) | ~10^4 PFU VEEV IAB strain TrD, 7 wpim | aerosol | PRNT80, 1 to ~4.5log<sub>10</sub> |
|                      |      |                              | New Zealand White rabbits, age not specified | 500 µg of VEEV<sub>CO</sub> i.m. electroporation | Three doses (0, 28 and 230 days) | ND | ND | PRNT80, ~3log<sub>10</sub> to 5log<sub>10</sub> | [184,185] |
|                      |      |                              | Cynomolgus macaques, age not specified | 50 or 500 µg of VEEV<sub>CO</sub> i.m. electroporation | Two doses (0 and 56 days) | 3 × 10^8 PFU VEEV IAB TrD | aerosol | PRNT80, ~0.8log<sub>10</sub> to 3.5log<sub>10</sub> |
|                      |      |                              | Human clinical trial, Phase 1 | 0.5 or 2 mg i.m. electroporation or i.d. electroporation | Three doses (days 0, 28, and 56) | NA | NA | GMT PRNT80, 7 to 78 |
| WEEV                 | pVHX-671V-1658 pVHX-6 CBA87 pVHX-6 Fleming | | BALB/c mice, age not specified | 2 shots × 2.5 µg precipitated on 0.5 mg gold | gene gun | Four doses (2 weeks apart) | 1.5 × 10^3 PFU WEEV Fleming, CBA 87 or 71V-1658, 8 wpim | i.n. | ND | [186] |
| WEEV and EEEV        | LANAC E1ecto | WEEV McMillan | CD-1 mice, 4 to 6 week old | 10 µg s.c. injection dorsal to the cervical spine | Two doses (2 weeks apart) | 10^4 PFU WEEV McMillan, Montana-64, or EEEV Florida-93, 4, 5, 9, 11, or 13 wpim | i.n. or s.c. | PRNT50, <40 to 200 | [187] |
### Table 1. Cont.

| Vaccine Against Virus | Name | Strain Vaccine Modelled After | Phase | Immunization | Challenge | Humoral Immune Response(s) | Ref |
|-----------------------|------|-----------------------------|-------|--------------|-----------|---------------------------|-----|
| CHIKV                | mRNA-1388 (or VAL-181388 in clinical trials) | NA | Human clinical trial, Phase 1 | 25, 50 or 100 µg, i.m. | Dose escalation procedure (0 and 4 weeks) | ND | ND | 'dose-dependent increase' in neutralizing and binding antibody titers | [188] |
| CHIKV                | mRNA-1944 SL15649 | AG129, age not specified | Cynomolgus macaques, 2 to 3 year old | 0.4, 1 or 10 mg/kg, i.v. tail vein injection | Single dose, 10^3 TCID50 of CHK subcutaneous injection in the footpad and hock of the right leg | ND | ND | FRNT50, 5 to 12 | [189,190] |
|                      |      | Human clinical trial, Phase 1 (active, not recruiting) | | 0.1, 0.3 and 0.6 mg/kg, i.v. | Dose escalation | NA | NA | NT50, 'all participants also showed circulating neutralizing antibody activity' | |
| Subunit              |      | CHIKV-sE1 and -sE2 | S27 | AG129 mice, 6 week old | 2 µg, s.c. | Two doses (0 and 21 days), 1000 TCID50 of S27 isolate, 9 vpm | i.p. | NT95, <25 | [164,165, 191] |
|                      |      | CHIKV rE2p | IND-06-AP3 | BALB/c, 6 to 8 week old | 10, 20 or 50 µg, i.m. | Two doses (2 weeks apart), Mice immunized with 50 µg challenged with 7 log10 TCID50 /mL, 5 or 22 vpm | i.n. | NT50, 0.25 log10 to 2.5 log10 | [154] |
|                      |      | CHIKV CHIK Eve1 and CHIKE2 recombinant proteins | DRDE-06 | BALB/c | 40 µg, s.c. | Three doses (0, 21 and 35 days) | ND | ND | PRNT90, 32 to 512 | [192] |
### Table 1. Cont.

| Vaccine Against Virus | Name          | Strain Vaccine Modeled After | Phase              | Immunization | Challenge | Humoral Immune Response(s) | Ref        |
|-----------------------|---------------|-----------------------------|--------------------|--------------|-----------|---------------------------|------------|
|                       |               |                             |                    | Dose         | Route     | Schedule                  |            |
|                       |               |                             |                    | Route(s)     | Dose (Strain, Genotype) | Route     |                           |            |
| **Chimeric virus**    |               |                             |                    |              |           |                           |            |
| **Measles virus-based chimeras** | | | | | | | |
| CD46-IFNAR, 6 week old | MV-CHIKV | 06–49                       |                    | 10^3 to 10^5 TCID50 | i.p. | Single or two doses (30 days apart) | 100 PFU of CHIKV 06-49, 2 mpim | i.p. | PRNT50, 450 to 4050 PRNT90, 50 to 450 |
| Cynomolgus macaques, age not specified | | | | 5 × 10^5 (± 0.5 log) TCID50 | i.m. | Two doses (28 days apart) | 1.4 × 10^5 PFU LR2006 OPY1, 56 dpim | s.c. | PRNT80, 40 to >640 |
| Human clinical trial, Phase 1 | | | | 1.5 × 10^4, 7.5 × 10^3 or 5.3 log TCID50 | i.m. or s.c. | Dose escalation (0 and 28 days, or 0 and 90 days) | NA | NA | PRNT50, 5 to 433 |
| Human clinical trial, Phase 2 | | | | 5 × 10^4 or 5 × 10^5 TCID50 | i.m. | Three doses (0, 28, and 196 days) | NA | NA | PRNT50, ~5 to 5000 |
| **Alphavirus-based chimeras** | | | | | | | |
| CHIKV | VEE/CHIKV EEE/CHIKV SIN/CHIKV | LR2006 OPY1 | NIH Swiss, C57BL/6, >3 week old | 5.8 log_{10} PFU (VEE/CHIKV and SIN/CHIKV), 5.3 log_{10} PFL (EEE/CHIKV) | s.c. in the medial thigh | Single dose | 6.5 log_{10} PFU (Ross CHIKV strain), 21 dpim | i.n. | PRNT80, 20 to 320 |
| CHIKV | VEE/IRES-CHIKV VEE/IRES-C/CHIKV | | A129 mice, 6 to 9 week old | 10^4 PFU | s.c. | Single dose | 10^5 PFU of LR2006 OPY1, 5 weeks post immunization | s.c. | PRNT80, >640 |
| CHIKV | EILV-CHIKV | CHIKV 996659 | C57BL/6 mice, 4 week old | 8.8 log_{10} PFU | s.c. | Single dose | 6 log_{10} PFU 99659, 30 dpim | i.d. | PRNT80, ≥ 80 |
| Cynomolgus macaques, 3 to 5 years | 8.1 log_{10} PFU | i.m. into the right quadriceps | Single dose | 5 log_{10} PFU LR2006 OPY1, 31 dpim | s.c. | PRNT80, 80 to 640 |
| Vaccine Against Virus | Name     | Strain Vaccine Modelled After | Phase                                      | Immunization | Challenge                                      | Humoral Immune Response(s) | Ref         |
|-----------------------|----------|------------------------------|--------------------------------------------|--------------|-----------------------------------------------|----------------------------|-------------|
|                       |          |                              |                                            | Dose Route Schedule | Dose (Strain, Genotype) Route                  |                            |             |
| EEEV                  | EILV/EEEV| EEEV FL-93                   | Adult CD-1 mice (age not specified)       | 10^6 PFU s.c. Single dose | 10^5 PFU EEEV-FL93, 70 dpim i.p.              |                            | [125,220]  |
| EEEV                  | Trivalent| EEEV FL-93, VEEV IAB TC-83, CHIKV 996659 | Adult CD-1 mice (age not specified)       | 10^6 PFU s.c. Single dose | 10^5 PFU EEEV-FL93, 70 dpim i.p.              |                            |             |
| VEEV                  | EILV/EEEV| VEEV IAB TC-83               | Adult CD-1 mice (age not specified)       | 10^6 PFU s.c. Single dose | 10^5 PFU VEEV-IC 3908, 70 dpim s.c.          |                            | PRNT80, 80 to 1280 |
| VEEV                  | Trivalent| EEEV FL-93, VEEV IAB TC-83, CHIKV 996659 | Adult CD-1 mice (age not specified)       | 10^6 PFU s.c. Single dose | 10^5 PFU VEEV-IC 3908, 70 dpim s.c.          |                            | PRNT80, 40 to 640 and 20 to 640 for mono- and trivalent vaccines respectively |
| EEEV                  | SIN/NAEEEV| EEEV FL-93-939              | NIH Swiss mice, 8 week old                | 3.7, 4.7 or 5.7 log_{10} PFU s.c. Single dose | 6 log_{10} PFU FL-93-939, 28 dpim i.p.      |                            | PRNT80, 125 to 660  |
| SIN/SAEEEV            | EEEV     | EEEV BeAr436087             | NIH Swiss mice, 8 week old                | 3.8, 4.8 or 5.8 log_{10} PFU s.c. Single dose | 6 log_{10} PFU FL-93-939, 28 dpim i.p.      |                            | PRNT80, 28 to 308 |
| SIN-83                | VEEV IAB | VEEV IAB TC-83               | Weanling NIH Swiss mice, 6 day old        | 10^3, 10^4, 10^5 or 10^6 PFU s.c. Single dose | 10^5 PFU VEEV IC ZPC738 IC SH3 s.c. in medial thigh |                            | PRNT80, 30 to 960 |
|                       |          |                              | NIH Swiss mice, 6 week old                | 5 x 10^5 PFU s.c. Two doses | 2 x 10^6 or 10^7 PFU VEEV ZPC738, 8 wpim s.c., i.c., or i.n. |                            | PRNT80, 55 to 73 (single), 100 to 160 (booster) |
| VEEV                  | SAAR/TRD | VEEV IAB TrD                | NIH Swiss mice, 6 week old                | 5 x 10^5 PFU s.c. Two doses | 2 x 10^6 or 10^7 PFU VEEV ZPC738, 8 wpim s.c., i.c., or i.n. |                            | PRNT80, 126 to 167 (single), 152 to 160 (booster) |
| SIN/TRD               | VEEV IAB | VEEV IAB TrD                | NIH Swiss mice, 6 week old                | 5 x 10^5 PFU s.c. Two doses | 2 x 10^6 or 10^7 PFU VEEV ZPC738, 8 wpim s.c., i.c., or i.n. |                            | PRNT80, 37 to 57 (single), 50 to 73 (booster) |
| SIN/ZPC               | VEEV ID  | ZPC738                      | NIH Swiss mice, 6 week old                | 5 x 10^5 PFU s.c. Two doses | 2 x 10^6 or 10^7 PFU VEEV ZPC738, 8 wpim s.c., i.c., or i.n. |                            | PRNT80, 187 to 253 (single), 253 to 487 (booster) |
| All the above         | VEEV IAB | VEEV IAB TC-83, IAB TrD, ID ZPC738 | Syrian golden hamsters, 6 week old        | 5 x 10^5 PFU s.c. in the medial thigh Single dose | 10^6 PFU s.c. in medial thigh                 | ND                         |             |
### Table 1. Cont.

| Vaccine Against Virus | Name | Strain Vaccine Modelled After | Phase | Vaccine | Immunization | Challenge | Humoral Immune Response(s) | Ref |
|-----------------------|------|-------------------------------|-------|---------|--------------|-----------|---------------------------|-----|
| **WEEV**              |      |                               |       |         |              |           |                           |     |
| SIN/C092              | WEEV | NIH Swiss mice, 6 week old    |       |         | 3.5, 4.5, or 5.0 log<sub>10</sub> PFU | s.c. in the medial thigh | Single dose | 5.3 log<sub>10</sub> PFU WEEV TBT235, 28 dpim | i.n. | PRNT80, 20 to 640 [203] |
| SIN/SIN/McM           | WEEV | NIH Swiss mice, 6 week old    |       |         | 4.8 or 5.8 log<sub>10</sub> PFU | s.c. in the medial thigh | Single dose | 5.0 log<sub>10</sub> PFU WEEV McMillan, 28 dpim | i.n. | PRNT80, 600 to 604 |
| SIN/EEE/McM           | EEEV | NIH Swiss mice, 6 week old    |       |         | 4.6 or 5.6 log<sub>10</sub> PFU | s.c. in the medial thigh | Single dose | 5.0 log<sub>10</sub> PFU WEEV McMillan, 28 dpim | i.n. | PRNT80, 416 to 420 |
| **Vacccinia virus-based chimeras** |      |                               |       |         |              |           |                           |     |
| CHIKV                 | MVA-CHIKV | C57BL/6 mice, 6 to 8 week old |       |         | 10<sup>7</sup> PFU (first dose), 2 × 10<sup>7</sup> PFU (second dose) | i.p. | Two doses (2 weeks apart) | 10<sup>6</sup> PFU LR2006-OPY1, 9 wpim | s.c. in the dorsal side of each hind foot | NT50, –100 to 3000 [204] |
| CHIKV                 | MVA-CHIK | BALB/c mice, 4 to 6 week old  |       |         | 10<sup>7</sup> TCID50 units | i.d. injection into the left hind footpad. | Single or two doses (28 days apart) | 10<sup>4</sup> LR2006 OPY1 TCID50 units at 39 or 42 dpim | i.d. | TCID50, 5 to 15 [205] |
| CHIKV                 | MVA-6KE1, MVA-EEE2, MVA-6KE1E3E2 | AG129, 6 to 10 week old |       |         | 10<sup>7</sup> TCID50 units | i.d. injection into the left hind footpad. | Single or two doses (28 days apart) | 10<sup>2</sup> LR2006 OPY1 TCID50 units at 39 or 42 dpim | i.d. | TCID50, 4 to 8 |
| EEEV, VEEV, and WEEV  |      | BALB/c mice, age not specified |       |         | 5 × 10<sup>6</sup> TCID50 | i.m. into the quadriiceps muscles of the left leg | Two doses (3 weeks apart) | 10<sup>3</sup> TCID50 CHIKV-S27 and CHIKV-IND/NL10, 63 dpim | i.p. | NT100, 10 to 160 [206] |
|                        |      |                               |       |         |              |           |                           |     |

**Note:** PFU = plaque forming units; TCID50 = 50% tissue culture infectious dose; NT50 = 50% neutralization titer.
| Vaccine Against Virus | Name | Strain Vaccine Modelled After | Phase | Immunization | Challenge | Humoral Immune Response(s) | Ref |
|-----------------------|------|------------------------------|-------|--------------|----------|---------------------------|-----|
| Adenovirus-based chimeras | | | | | | | |
| CHIKV | CAdVax-CHIK | LR2006 OPY1 | CD-1 or C57BL/6, 6 to 8 week old | 10^8 IU | i.p. | Single dose | 10^4 CCID50 LR2006 OPY1 or QIMR, 6.5 wpim | s.c. into side of each hind foot towards the ankle | NT100, ~2000 [208] |
| CHIKV | ChAdOx1 Chik | | BALB/c, 6 to 8 week old | 10^8 IU | i.m. | Single dose | ND | ND | NT50, 5.39 × 10^3 [209,210] |
| | ChAdOx1 Chik ∆Cap | | AG129, 5 week old | 10^8 IU | i.m. in each leg | Single dose | 9.7 × 10^4 PFU LR2006 OPY1, 30 dpim | i.d. into the left foot | ND |
| CHIK001 (in clinical trials) | | | Human clinical trial, Phase 1 | 5 × 10^9, 2.5 × 10^10 or 5 × 10^11 vp | i.m. | Single dose | 1.6 × 10^4 PFU MAYV-CH, 30 dpim | i.d. into the left foot | PRNT50, 160 to 620 [210] |
| MAYV | ChAdOx1 May | | AG129, 5 week old | 1.6 × 10^4 PFU | i.m. in each leg | Single dose | 1.6 × 10^4 PFU MAYV-CH, 30 dpim | i.d. into the left foot | PRNT50, 160 to 620 [210] |
| VEEV | Rad/VEEV#3 | VEEV IAB TC-83 | BALB/c, 6 to 8 week old | 10^7 PFU | i.n. | Three doses (at 0, 7 and 21 days) | Dose ND, 28 dpim aerosol | PRNT50 (NP) [213] |
| | | | BALB/c, 6 to 8 week old | 10^7 PFU | i.n. | Two doses (at 0, 21 days) | 5000 LD50 TrD, 42 dpim aerosol | ND | [214] |
| WEEV | Ad5-VEEV | WEEV 71V-1658 | BALB/c mice, age not specified | 10^7 PFU | i.m. | Single or two doses (at 4 weeks) | 1.5 × 10^3 PFU Fleming or 71V-1658, 13 wpim | i.n. | PRNT50, 160 [215] |
| WEEV | Ad5-E1 | WEEV 71V-1658 | BALB/c mice, 6 to 9 week old | 10^7 PFU | i.m. in both leg | Single dose | 50 LD50 of 71V-1658, 7 dpim, or 400 LD50 CBA87, 1, 3, 5 or 7 dpim | i.n. | PRNT50, <10 [216] |
| Vesiculovirus-based chimeras | | | | | | | |
| CHIKV | rVSVΔG-CHIKV | CHIKV S27 | C57BL/6, 3 week old | 10^6 PFU | i.m. into the right hind leg muscle | Single dose | 10^4 PFU LR 2006 OPY1, 30 dpim | s.c. in the left rear footpad | PRNT80, 80 to 640 [217] |
Table 1. Cont.

| Vaccine Against Virus | Name   | Strain Vaccine Modelled After | Phase | Immunization | Challenge | Humoral Immune Response(s) | Ref |
|-----------------------|--------|------------------------------|-------|--------------|----------|-----------------------------|-----|
|                       |        |                              |       | Dose | Route | Schedule | Dose (Strain, Genotype) | Route |                     |
| VEEV                  | rSVSIV-VEEV | VEEV ZPC738                 | CD-1, 4 to 6 week old | 10⁵ / 10⁷ PFU | i.m. | Single dose | 10⁴ PFU VEEV ZPC738, 35 or 245 dpim | s.c. | PRNT80, 288 to 600 at 25 and 35 dpim, 304 to 360 at 245 dpim | [218] |
| VEEV                  | rISFV-VEEV | VEEV ZPC738                 | CD-1, 4 to 6 week old | 10⁸ PFU | i.m. | Single dose | 10⁴ PFU VEEV ZPC738, 28 dpim | s.c. | PRNT80, ≥20 |
|                       |        |                              | CD-1, 4 to 6 week old | 10⁸ PFU | i.m. | Single dose | 10⁴ PFU VEEV ZPC738, 35 or 245 dpim | s.c. | PRNT80, 40 to 160 at 25 and 35 dpim, 25 to 64 at 245 dpim |
| EEEV                  | rISFV-EEEV | EEEV FL93-939               | CD-1, 4 to 6 week old | 10⁸ PFU | i.m. | Single dose | 10⁴ PFU EEEV FL93-939, 28 dpim | s.c. | PRNT80, ≥20 |
| Epitope-based         |        |                              |       |      |        |          |                           |      |                     |
| CHIKV                 | E2EP3  | NA                          | C57BL/6 mice, 3 week old | 100 µg (50 µg for booster doses) | s.c. in the abdominal flank | Three doses (0, 14 and 21 days) | 10⁶ PFU CHIKV SGP11, 30 dpim | s.c. region at the ventral side of the right hind footpad, towards the ankle | ~40% reduction from mock control | [23] |

1 s.c., subcutaneous; i.v., intravenous; i.m., intramuscular; i.d., intradermal; i.p., intraperitoneal; i.n., intranasal; i.t./i.s., intrathalamic/ intraspinal; i.pl., intraplantar; i.c., intracranial; dpim, days post immunization; wpim, weeks post immunization; mpim, months post immunization; IRES, internal ribosome entry site; PFU, plaque forming units; TCID₅₀, 50% tissue culture infective dose; CCID₅₀, 50% cell culture infectious dose; IC₅₀, 50% inhibitory concentration; GE, genomic equivalents; IU, infectious units; AID₅₀, 50% animal infectious dose; PRNT₅₀, 50% plaque reduction neutralizing antibody titer; PRNT₉₀, 90% plaque reduction neutralizing antibody titer; LD₅₀, median lethal dose; NT₅₀, 50% neutralizing titer; GMT, geometric mean titer; µNT, neutralizing titer; SIN, Sindbis virus; ISFV, Isfahan virus; May, Mayaro virus; EIILV, Eilat virus, VSV/VSIV, vesicular stomatitis virus; MV, measles virus; MVA, modified vaccinia virus Ankara; NP, not provided; NA, not applicable; WT, wild type. Data curated from literature reported through February 2021.
3.1. Live-Attenuated Vaccines

With the development of alphaviruses in reverse genetic systems, more research has been focused on the rational design of live-attenuated vaccines [221,222] in overcoming potential issues, such as genetic reversion mutations in vaccines [223,224], with highly specific mutations or alterations of the original parental virus genome. In addition, not only are the safety profiles of these vaccines greatly improved, protection with a single dose is also achieved [225].

An engineered live-attenuated option for alphavirus vaccine design involves the rational design of downregulating the expression of particular structural proteins with the introduction of a picornavirus (encephalomyocarditis virus) internal ribosome entry site (IRES) into the viral genome. For example, this is demonstrated in a VEEV vaccine candidate, ZPC/IRES, where the expression of the capsid protein is minimalized by translocating its gene to a separate opening reading frame downstream of the envelope glycoprotein genes and interrupting its expression with the introduction of a IRES [114,226]. However, the highly immunogenic envelope glycoproteins E3-E1 were not manipulated, but the insertion of IRES into the genome would functionally alter the host range as replication of the live virus is restricted in mosquitoes.

ZPC/IRES is based on a full-length clone of a wild type VEEV subtype ID from Zulia state, Venezuela from a sentinel hamster exposed in a tropical lowland. CD-1 mice immunized with $10^5$ PFU of ZPC/IRES developed strongly neutralizing antibodies, with PRNT80 of average reciprocal titer of 324 by 20 weeks post immunization. Subsequently, when immunized mice were challenged with the lethal VEEV subtype IC strain 3908 ($10^5$ PFU, subcutaneous or $10^4$ PFU, aerosol route) 4 weeks after immunization, all mice retained their weight and failed to show any signs of disease and survived, compared to mock-vaccinated mice which succumbed to the lethal infection. Additionally, the study tested the vaccine in a NHP immunization-challenge model in the same study. Vaccinated NHPs had PRNT80 values of 160 to 320, and all vaccinated NHPs were protected against viremia upon challenge with VEEV 3908 strain. Using the VEEV ID strain ZPC738 as the vaccine backbone, which is closely related to subtypes IAB and IC, the authors had aimed to develop an IRES-based, live-attenuated vaccine candidate that could possibly protect against other subtypes of VEEV, given that previous attempts to create a vaccine candidate based on the VEEV subtype IAB V3526 vaccine could not significantly protect against aerosol challenge with a subtype IE VEEV strain [226]. Nonetheless, this hypothesis was not pursued in the study, and it would have been curious to learn whether the ZPC/IRES-immunized animals would be protected from a lethal challenge with VEEV of other subtypes, such as subtypes IA/B and IE.

3.2. Inactivated Vaccines

The inactivated Ross River virus (RRV) vaccine is the most developed and advanced vaccine candidate, having been rigorously tested in both preclinical and up to Phase 3 clinical trials. The Vero cell culture-derived whole-virus RRV vaccine was first produced from a viral seed derived from an RRV isolate from a serologically confirmed case of RRV disease in Queensland, Australia, and subsequently inactivated by sequential formalin and UV light treatment after harvest [227]. In pre-clinical testing of the RRV vaccine, CD-1 mice were given two doses of the inactivated RRV vaccines at different experimental doses 28 days apart, without the use of an adjuvant in its formulation. Upon challenge with $10^6$ TCID50 of the mouse-virulent RRV prototype strain T48 at 42 days post immunization, a vaccine dose beyond 0.625 $\mu$g provided almost complete protection against viremia development at 1-day post challenge. Interestingly, the possible antibody-dependent enhancement by RRV vaccination by a closely related alphavirus infection was investigated, where viremia in CHIKV LR2006 OPY-1-infected-RRV vaccinated mice was significantly reduced as compared to the control. In this heterologous situation, partial cross protection was observed, but the presence of sub-protective levels of RRV vaccine-induced antibodies prevented the enhancement of CHIKV replication [73].
Subsequently, a randomized Phase 3 clinical trial for the RRV vaccine was conducted in Australia to investigate the safety and immunogenicity of the vaccine in a large cohort of 1755 healthy younger adults aged 16 to 59 years and 209 healthy older adults aged > 60 years [115]. The 2.5 \( \mu \)g Al(OH)3-adjuvanted vaccine was given over three doses (subsequent boosts at 3 weeks and 6 months). The majority of participants in the younger and older adult populations had seroprotective uNT titers after three immunizations with the whole-virus RRV vaccine, and titers of serum IgG antibodies after three immunizations were higher than the serological IgG ELISA titer threshold associated with protection after natural infection with RRV [115]. While the RRV vaccine had been brought forward to Phase 3 clinical trials, and despite the vaccine demonstrating safety and efficacy, it was not considered financially viable to manufacture, despite Queensland recording its largest and worst epidemic between 2014 to 2015 [228,229]. In addition, given that the cost of vaccine trials is hard to justify for a disease that occurs only in Australia and Papua New Guinea, and where the disease is never fatal, efforts to further develop the RRV vaccine were unfortunately halted.

3.3. Virus-Like Particles (VLPs)

The VRC-CHKVLP059-00-VP is one of the first potential new CHIKV vaccines to reach advanced development with human clinical testing [116,117]. The CHIKV envelope gene cassette encoding the native polypeptide, E3-E2-6K-E1, of CHIKV strains 37,997 (West African genotype) and LR2006 OPY-1 were inserted into a cytomegalovirus CMV/R expression vector and subsequently transfected into 293T human kidney cells [118]. The resulting VLP product is a CHIKV VLP that is structurally identical to its infectious counterpart (given that structural genes are intact), but is not infectious as its genetic material is removed. While the CHIKV 37,997 strain yielded approximately 100 times more VLPs than that from strain LR2006 OPY-1, the former strain was subsequently used to produce the VLPs. Nonetheless, given that the ECSA lineage was responsible for the ongoing outbreak at the time of development, the high degree of amino acid similarity between the two CHIKV strains suggested that the vaccine would be protective against viruses of other genotypes. However, it would have been curious to characterize a VLP produced from a CHIKV strain of the ECSA lineage, given that it is the strain responsible for recent Chikungunya epidemics all around the world [119–123].

BALB/c mice immunized with two doses of 19 \( \mu \)g of CHIKV VLPs intramuscularly generated the highest neutralizing titer against both the homologous strain 37,997 and the heterologous strain LR2006 OPY-1. In addition, NHPs immunized with 20 \( \mu \)g of VLPs developed substantial neutralizing activity to both homologous and heterologous strains after primary immunization. Interestingly, even though the VLP was made from CHIKV 27,997 strain, there was slightly better neutralization of LR2006 OPY-1 compared to 37,997 in both mice and NHPs. The study speculated that this is suggestive that the LR2006 OPY-1 virus may present a conserved epitope to the immune system better than the 37,997 virus. When total IgG antibodies were passively transferred from immunized NHPs to defective type 1 interferon signaling immunodeficient mice (Ifnar1\(^{-/-}\)), these recipient mice did not develop detectable viremia and all survived a lethal challenge with CHIKV LR2006 OPY-1. This indicated that the humoral immune responses induced by the CHIKV VLPs confer protection against CHIKV infection [124].

This promising data eventually led to further testing in clinical trials—phase 2 studies were concluded and reported in 2020 [117]. The randomized phase 2 clinical trial included 400 healthy adults in outpatient clinics in 6 countries in the Caribbean. Two doses of 20 \( \mu \)g of CHIKV VLP, termed as VRC-CHKVLP059-00-VP in clinical trials, were administered 28 days apart via intramuscular injection. Vaccine-induced humoral immune responses in individuals were comparable with titers from participants vaccinated in the phase 1 trial [116], and serum collected from participants in the phase 1 trial induced neutralizing antibodies against all 3 genotypes of CHIKV. Interestingly, while the phase 2 trial aimed to only enroll CHIKV seronegative participants, 20% of the cohort (in particular, participants
from 2 study sites—Dominican Republic and Haiti) were retrospectively found to be seropositive at baseline on the day of the study enrolment, possibly due to seroconversion between screening and study enrolment. A post-hoc analysis demonstrated that the VLP was immunogenic among these seropositive recipients, but a significant difference was observed between the geometric mean ratio between seropositive and seronegative vaccine recipients. Further studies on this specific group of participants to understand the possible effects of seropositivity and efficacy or protection of the CHIKV VLP administered will be interesting, rendering the need for additional clinical trials [117].

3.4. Chimeric Viruses

Another option in vaccine development in providing high levels of immunity is the use of a virus-vector system that utilizes an avirulent backbone, but incorporates the expression of viral genetic elements, such as the chimeric vector system for producing foreign gene products.

The insect-only host-restricted Eilat virus (EILV) has recently also been utilized as a chimeric backbone to replace the structural open reading frame with that of EEEV, VEEE or CHIKV [125]. Given that EILV is unable to replicate in vertebrate cells and in brain tissues of infant mice, this enhances the safety aspect of the vaccine, and thus, also serves as a inactivated vaccine, which enhances the expression of particular immunogenic proteins. Separately, the monovalent EILV/EEEV and EILV/VEEV vaccines were efficacious in their protection against lethal alphavirus challenge—immunized CD-1 mice had a high seroconversion rate observed post vaccination and were highly protected from lethal EEEV-FL93 or VEEV-3908 challenge. Compared to mock-vaccinated animals, EILV/EEEV or EILV/VEEV immunized animals had little or no weight loss and were protected from disease. More importantly, a trivalent vaccine containing the EILV/EEEV, EILV/VEEV and EILV/CHIKV chimeras was formulated and assessed if the vaccine could provide protection against lethal challenge with multiple alphaviruses. A single trivalent dose of EILV/VEEV, EILV/EEEV, and EILV/CHIKV elicited neutralizing antibodies against all three viruses and provided >80% protection against VEEV and EEEV lethal challenge. Collectively, this work showed safety combined with strong immunogenicity and ease of production, making the use of the EILV alphavirus chimeric vaccine platform promising and attractive [125]. The use of a trivalent vaccine candidate also serves as a proof-of-concept to show practicality and increases its potential as a vaccine against neurotropic alphaviruses.

3.5. Nucleic Acid-Based Vaccines

Commonly known as the ‘third-generation vaccine’, RNA and DNA vaccines form one of the latest vaccine approaches for alphaviruses. The risk of infection from receiving a vaccine is minimal, given the safety associated with the nucleic acid product [230]. In addition, as some vaccines have been shown to have poor immunogenicity due to the lack of uptake or the need for adjuvants [231], much research over the past decades have explored the design of constructs and novel delivery technologies to overcome these issues. In order to overcome several issues related to traditional vaccine development, such as high cost and difficulty in production, RNA has emerged as an effective platform to deliver vaccines using nanoparticle delivery vehicles, such as liposomes [232–234].

A RNA vaccine against CHIKV involves the delivery of the self-replicating RNA genome of the live attenuated CHIKV-NoLS virus with CAF01 liposomes [126]. The mutation in the nucleolar localization sequence (NoLS) in the capsid protein of CHIK-NoLS was previously shown to significantly attenuate viral replication [127]. In the same study, C57/BL6 mice immunized with one dose of CHIKV-NoLS were fully protected from CHIKV infection [127]. In immunodeficient AG129 mice, a single dose of CHIKV-NoLS RNA delivered with CAF01 generated CHIKV-specific neutralizing antibodies. While these immunized AG129 mice developed disease signs, they eventually recover from the immunization, compared to mock-immunized mice. Importantly, CHIKV-NoLS CAF01-immunized AG129 mice survive from subsequent CHIKV challenge and do not develop
CHIKV-induced footpad swelling or disease. On the other hand, in immunocompetent C57/BL6 mice, CHIKV-NoLS CAF01-immunized mice developed delayed viremia at a similar titer compared to CHIKV-WT, and were protected from footpad swelling. However, immunization with either CHIKV-NoLS CAF01 or CHIKV-NoLS RNA produced significantly lower levels of neutralizing antibody compared to CHIKV-WT inoculation [126]. However, this study showed that the RNA-launched self-assembling viral particles generated immunity and protection that were just as strong as those of wild-type viral particles, suggesting the significant potential of this approach.

While multiple novel approaches have been explored to develop vaccines against alphaviruses, a potential prophylactic strategy that could be the development of a multivalent alphavirus vaccine given the reports of cross-neutralizing antibodies against conserved epitopes in the E2 protein across closely related alphaviruses. This approach would prove useful in endemic areas where alphavirus co-circulation occurs.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/microorganisms9050899/s1. Supplementary Figure S1. Alignment of E1 and E2 amino acid sequences of arthritogenic and encephalitic alphaviruses.

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