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Review article

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A molecular understanding of alphavirus entry and antibody protection

Arthur S. Kim [1,2] & Michael S. Diamond [3,4,5,6]

Abstract

Alphaviruses are arthropod-transmitted RNA viruses that cause epidemics of human infection and disease on a global scale. These viruses are classified as either arthritogenic or encephalitic based on their genetic relatedness and the clinical syndromes they cause. Although there are currently no approved therapeutics or vaccines against alphaviruses, passive transfer of monoclonal antibodies confers protection in animal models. This Review highlights recent advances in our understanding of the host factors required for alphavirus entry, the mechanisms of action by which protective antibodies inhibit different steps in the alphavirus infection cycle and candidate alphavirus vaccines currently under clinical evaluation that focus on humoral immunity. A comprehensive understanding of alphavirus entry and antibody-mediated protection may inform the development of new classes of countermeasures for these emerging viruses.

Sections

[1]Department of Chemistry, The Scripps Research Institute, La Jolla, CA, USA. [2]Department of Immunology and Microbiology, The Scripps Research Institute, La Jolla, CA, USA. [3]Department of Medicine, Washington University School of Medicine, Saint Louis, MO, USA. [4]Department of Pathology and Immunology, Washington University School of Medicine, Saint Louis, MO, USA. [5]Department of Molecular Microbiology, Washington University School of Medicine, Saint Louis, MO, USA. [6]The Andrew M. and Jane M. Bursky Center for Human Immunology and Immunotherapy Programs, Washington University School of Medicine, Saint Louis, MO, USA. e-mail: mdiamond@wustl.edu

Introduction

Alphaviruses are single-stranded, positive-sense RNA viruses of the *Togaviridae* family that cause worldwide outbreaks with substantial morbidity. These viruses are categorized into groups based on their genetic relatedness and clinical manifestations. Arthritogenic alphaviruses, including chikungunya virus (CHIKV), Ross River virus (RRV), O'nyong'nyong virus (ONNV), Barmah Forest virus (BFV), Mayaro virus (MAYV) and Sindbis virus (SINV), cause musculoskeletal disease that is characterized by fever, rash, arthralgia, myalgia, myositis and acute and chronic polyarthritis[1]. Encephalitic alphaviruses, including Eastern equine encephalitis virus (EEEV), Western equine encephalitis virus (WEEV) and Venezuelan equine encephalitis virus (VEEV), infect cells in the central nervous system and cause meningitis and encephalitis, often with long-term debilitating neurological sequelae[2,3].

Socioeconomic and ecological factors, including urbanization and climate change, have affected the geographical distribution of mosquito-borne alphaviruses and enabled their emergence and spread (Box 1)[4,5]. Alphaviruses are principally transmitted by *Aedes*, *Culiseta* and *Culex* mosquito species, which facilitates infection of a range of mammalian and avian hosts[5]. As an example, over the past two decades, CHIKV has caused large-scale epidemics in Africa and Southeast Asia. In 2005–2006, an outbreak occurred on La Réunion island, where 266,000 cases of CHIKV infection were reported[6]. Subsequent epidemics occurred in Africa and Asia, and CHIKV emerged and spread in the Caribbean islands and the Americas in 2013–2014, with millions of infections documented[7]. RRV and BFV are endemic and enzootic in Australia[8], and MAYV is emerging in Central and South America[9]. Encephalitic alphaviruses are a concern given their ability to cause severe neurological disease and their potential to be used in biological warfare[10]. A VEEV outbreak in South America in 1995 resulted in over 75,000 infected cases with 300 deaths[11]. Annual EEEV outbreaks occur in the United States, and the case-fatality rate is near 50%, although the number of human infections remains low. Despite their potential for epidemic spread and for causing severe disease, no specific countermeasures exist to combat or prevent alphavirus infections.

In this Review, we highlight recent advances in our understanding of alphavirus entry and antibody-mediated protection. As other reviews on these topics have been published, here we focus on new developments[12–14]. Technological advances, such as functional genomic screens and human monoclonal antibody isolation methods, have facilitated new mechanistic insights and development of strategies to combat emerging and re-emerging alphavirus infections.

Alphavirus infection cycle and structure

The alphavirus RNA genome (~12 kb) encodes four nonstructural proteins (nsP1, nsP2, nsP3 and nsP4) and five structural proteins (capsid, E3, E2, 6K/TF and E1)[15–17]. Together, these proteins mediate viral transcription, replication and host cell antagonism[18]. The surface of the 70-nm virion is composed of 80 trimeric E2–E1 heterodimer spikes arranged in $T = 4$ icosahedral symmetry[15,19,20] (Fig. 1). Each trimeric subunit can mediate viral attachment and entry, and E2 and E1 are the principal targets for neutralizing antibodies[15].

The E3 and E2 glycoproteins are synthesized as the precursor p62 protein, which is subsequently cleaved by furin[21,22]. The E3 glycoprotein is a chaperone for the folding of the other structural proteins and prevents premature conformational changes of the E2–E1 heterodimer during transit through the acidic environment of the secretory pathway[23–25]. Although the E3 glycoprotein is cleaved during the maturation process, for some alphaviruses (for example, SINV and CHIKV) it can remain bound to the mature E2–E1 heterodimer[15,20,26]. E3 dissociation also depends on the pH of the culture medium and the confluency of infected cells[24]. The E2 protein comprises three domains (A, B and C), with domain A situated towards the centre of the trimeric spike, domain B on the outermost tip of the spike and domain C proximal to the viral membrane[15]. The E2 protein has key roles in attachment to cellular receptors and is a primary target of neutralizing antibodies[20,27–35]. Although implicated in glycoprotein processing and in virion assembly and release, the precise role of the 6K/TF membrane protein is less well understood[17,36,37]. In addition, these transmembrane proteins have been excluded from the recombinant protein preparations used for structural analysis owing to their hydrophobic nature[15]. The E1 protein is a class II fusion protein that comprises three domains (I, II and III) and mediates viral membrane fusion via a hydrophobic peptide[15,38,39]. Although the fusion peptide is normally hidden beneath E2 domain B, upon exposure to low pH in the early endosome, the E1 protein undergoes rearrangement, which enables fusion peptide insertion into the host lipid membrane[38–41]. Upon endosomal membrane fusion, the nucleocapsid penetrates into

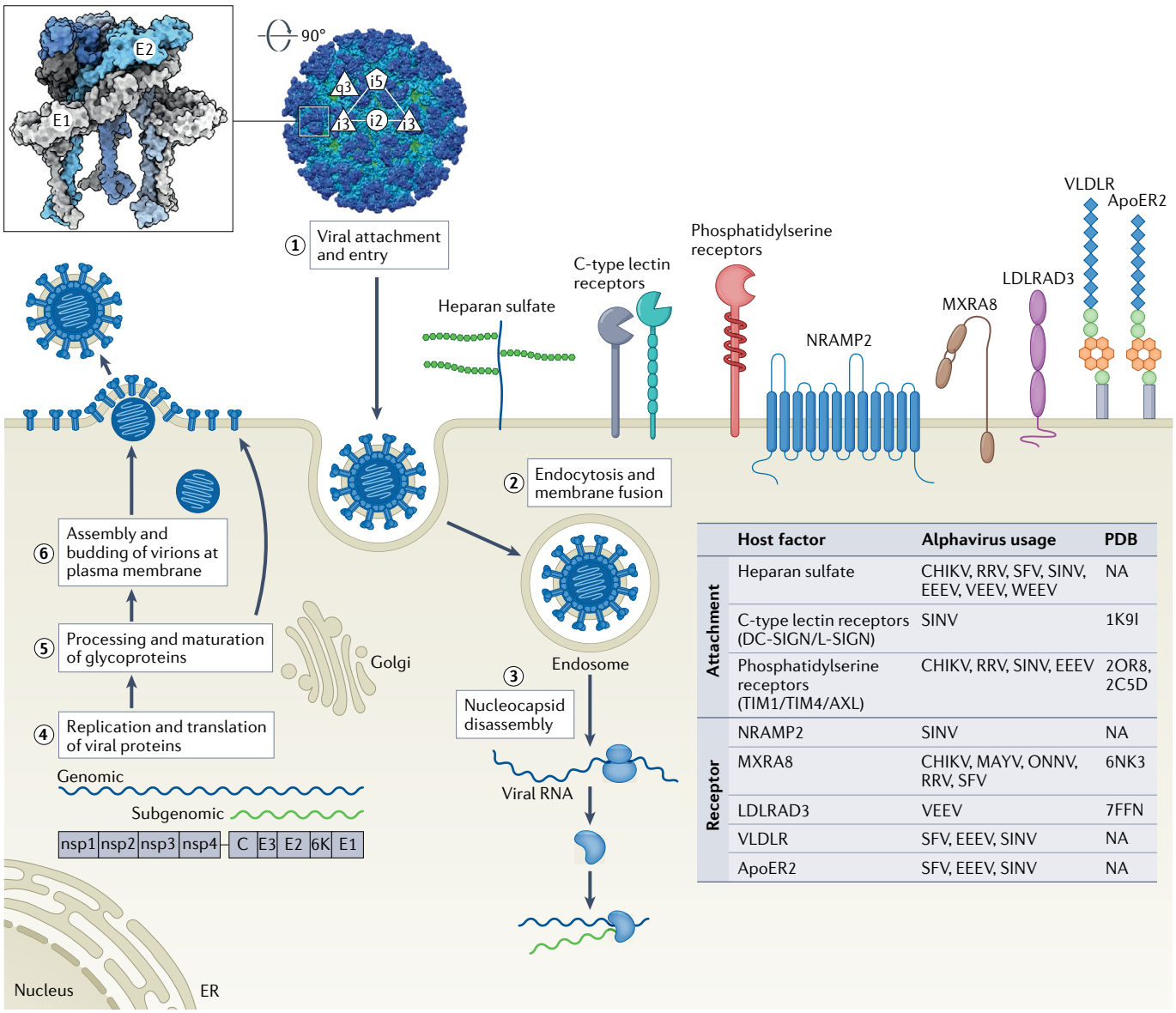
Box 1

Social and clinical impact of alphavirus infections

Owing to their epidemic outbreak potential, several alphaviruses including chikungunya virus (CHIKV), Eastern equine encephalitis virus, Venezuelan equine encephalitis virus and Western equine encephalitis virus are listed as Category B pathogens on the National Institute of Allergy and Infectious Diseases pathogen priority list. Alphaviruses are a global public health threat as reflected by the social and economic consequences that occur during outbreaks. The 2006 CHIKV epidemic on La Réunion island affected approximately one-third of the population and had an estimated economic burden of €43.9 million[165]. The economic burden from a subsequent CHIKV outbreak in the US Virgin Islands in 2014 ranged from US$14.8 million to $33.4 million, with a substantial fraction of individuals having long-term disability from CHIKV-induced chronic disease[166]. The emergence and re-emergence of alphavirus epidemics can be attributed in part to climate change, which enables increased vector-borne disease outbreaks and transmission[167]. Vector adaptation has also enhanced the spread and dissemination of alphaviruses as seen in the 2006 La Réunion island epidemic[168].

Alphaviruses are clinically distinguished by their ability to cause either arthritogenic or encephalitic disease. Acute clinical symptoms from arthritogenic alphavirus infection include fever, malaise, polyarthritis, myositis, myalgia and maculopapular rash[1,169]. Chronic disease can lead to persistent joint pain and inflammation. Encephalitic alphavirus infections can cause fever, meningitis, encephalitis and long-term neurological sequelae or death[3]. To date, treatment for alphavirus infections consists only of supportive care. Because of the disease alphaviruses cause and the potential for epidemic transmission, further studies to identify treatment and vaccine strategies against alphaviruses are warranted.

the cytoplasm, it disassembles, and genomic viral RNA is released and translated. The nonstructural proteins form a replication complex for subsequent synthesis of genomic and subgenomic viral RNA. The subgenomic RNA encodes viral structural proteins, which are translocated to the endoplasmic reticulum and transited to the Golgi complex where the glycoproteins undergo processing and maturation[18].



	Host factor	Alphavirus usage	PDB
Attachment	Heparan sulfate	CHIKV, RRV, SFV, SINV, EEEV, VEEV, WEEV	NA
	C-type lectin receptors (DC-SIGN/L-SIGN)	SINV	1K9I
	Phosphatidylserine receptors (TIM1/TIM4/AXL)	CHIKV, RRV, SINV, EEEV	2OR8, 2C5D
Receptor	NRAMP2	SINV	NA
	MXRA8	CHIKV, MAYV, ONNV, RRV, SFV	6NK3
	LDLRAD3	VEEV	7FFN
	VLDLR	SFV, EEEV, SINV	NA
	ApoER2	SFV, EEEV, SINV	NA

Fig. 1 | Alphavirus structure and entry mechanisms. The alphavirus virion (Protein Data Bank (PDB) ID: 6NK6) is composed of 80 trimeric E2–E1 heterodimer spikes (blue) arranged in $T = 4$ icosahedral symmetry. The fivefold (i5, pentagon), threefold (q3 and i3, triangle) and twofold (i2, circle) axes of symmetry are indicated on one icosahedral asymmetric unit (white triangle outline). An E2–E1 heterotrimer is shown in the zoomed left inset with E2 proteins depicted in light cyan, medium cyan and dark cyan, and E1 proteins depicted in light grey, medium grey and dark grey. Alphavirus entry is mediated by the E2 and E1 structural proteins through several attachment factors — namely, heparan sulfate, C-type lectin receptors (DC-SIGN[55] and L-SIGN) and phosphatidylserine receptors (TIM1 (ref.[163]), TIM4 and AXL[164]) — and receptors (NRAMP2 (ref.[64]), MXRA8 (refs.[20,35]), LDLRAD3 (refs.[67,68]), VLDLR[70] and ApoER2 (ref.[70])). Upon attachment, the alphavirus virion undergoes clathrin-mediated internalization (endocytosis) and subsequent membrane fusion in the endosome. The nucleocapsid is disassembled in the cytoplasm and releases viral RNA, which encodes four nonstructural proteins (nsP1, nsP2, nsP3 and nsP4) and five structural proteins (capsid, E3, E2, 6K/TF and E1). The viral nonstructural proteins are synthesized after translation of the input viral RNA to generate a replication complex for synthesis of additional genomic (blue) and subgenomic (green) viral RNA. The subgenomic viral RNA encodes the viral structural proteins, which are processed in the endoplasmic reticulum (ER) and Golgi complex and are subsequently transported to the plasma membrane for assembly and budding of virions. CHIKV, chikungunya virus; EEEV, Eastern equine encephalitis virus; MAYV, Mayaro virus; NA, not applicable; ONNV, O'nyong'nyong virus; RRV, Ross River virus; SFV, Semliki Forest virus; SINV, Sindbis virus; VEEV, Venezuelan equine encephalitis virus; WEEV, Western equine encephalitis virus. Virion diagram adapted with permission from ref.[20], Elsevier.

The newly synthesized genomic RNA is packaged with capsid proteins to form the nucleocapsid core[18]. The processed glycoproteins and the nucleocapsid are transported through either the secretory pathway or the cytopathic vacuole type II pathway to the plasma membrane, where virion assembly and budding occurs[42–44].

Alphavirus entry

Alphavirus entry into cells requires the engagement of attachment factors, receptors and endocytosis. The broad cellular tropism of alphaviruses is due in part to the utilization of multiple host factors during the attachment and entry process. Recently performed functional genomics and biochemical screens have provided new details as to how arthritogenic and encephalitic alphaviruses enter cells.

Alphaviruses can utilize multiple molecules to attach to the surface of host cells (Fig. 1). Heparan sulfate, a negatively charged glycosaminoglycan, interacts with positively charged amino acids in the E2–E1 heterodimer as described for CHIKV (E2–R82)[45,46], RRV (E2–R218)[47], SINV (E2–K70 and E2–R114)[48], EEEV (E2–K71, E2–K74 and E2–K74)[19,49,50] and VEEV (E2–K76, E2–K120 and E2–K209)[51]. Alphavirus dependence on heparan sulfate for binding occurs naturally or as an adaptation to cell culture passage[45,52]. The acquisition of positively charged amino acids that enable heparan sulfate binding increases viral infection in vitro but generally reduces pathogenesis in vivo[51,53] except for EEEV, for which heparan sulfate binding can increase neurovirulence in mice[49]. C-type lectins, including dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) and liver/lymph node-specific intracellular adhesion molecules-3 grabbing non-integrin (L-SIGN), are other reported attachment factors for alphaviruses. SINV produced in mosquito cells, which skews glycosylation to high-mannose N-linked oligosaccharides, shows greater binding to cells expressing DC-SIGN and L-SIGN[54], which is consistent with their preferential binding to proteins displaying high-mannose glycans[55]. Phosphatidylserine receptor proteins, such as TIM1, TIM4 and AXL, promote alphavirus attachment through interaction with phosphatidylserine on the viral membrane[56,57]. However, the extent to which these phosphatidylserine receptors enhance alphavirus attachment and infection is dependent on virus and cell type, as differences in utilization were observed in macrophages, keratinocytes and hepatocytes[56,58]. Further studies are warranted to elucidate the role of phosphatidylserine receptor proteins as alphavirus attachment factors and their contributions to pathogenesis.

The discovery of bona fide entry receptors for alphaviruses has historically been challenging. Although several putative receptors for alphaviruses have been identified using biochemical methods and genome-wide cDNA screens, direct physical binding to virions or effects of genetic ablation of the host factor on infection have not been demonstrated (Fig. 1). The laminin receptor was identified as an entry factor for SINV using a panel of antibodies reactive to host proteins[59]. Subsequent biochemical studies also implicated the laminin receptor as a receptor for VEEV[60], and similar methods have been utilized to identify other possible host receptors for alphaviruses including prohibitin and CD147 for CHIKV[61,62] and MHC class I for Semliki Forest virus (SFV)[63]. Application of a genome-wide RNA interference screen in *Drosophila* cells identified the metal ion transporter NRAMP and, subsequently, the mammalian orthologue NRAMP2 as receptors for SINV[64]. NRAMP2 was required for binding and infection by SINV but not for RRV. Gene editing of NRAMP2 in vitro and dNRAMP in *Drosophila* reduced SINV infection, establishing the role of these proteins in SINV infection in vitro and in vivo.

Recent advances with CRISPR–Cas9-based genome-wide screening have enabled the identification of several host receptors that facilitate alphavirus infection and pathogenesis (Fig. 1). Mouse MXRA8, a dual-immunoglobulin-like domain molecule, was identified as a receptor for multiple arthritogenic alphaviruses including CHIKV, MAYV, ONNV, RRV and SFV, but not for encephalitic alphaviruses or SINV[35]. Deletion of MXRA8 or its human orthologue reduced infection by arthritogenic alphaviruses. Direct binding was observed between MXRA8 and CHIKV, which was abrogated by anti-MXRA8 monoclonal antibodies (mAbs), anti-CHIKV mAbs or soluble MXRA8-Fc decoy molecules. X-ray crystallography and cryo-electron microscopy (cryo-EM) studies confirmed the MXRA8–CHIKV interaction and revealed a unique binding mode with both domains of MXRA8 engaging the E2 and E1 proteins on the alphavirus virion[20,34]. Through use of MXRA8-Fc decoy molecules and gene-edited mice, MXRA8 was shown to have a key role in arthritogenic alphavirus infection and disease pathogenesis in vivo[35,65,66].

A separate CRISPR–Cas9 screen identified the low-density lipoprotein (LDL) family scavenger receptor LDLRAD3 as a receptor for VEEV[67]. Gene editing of LDLRAD3 abrogated infection of VEEV but not of EEEV or WEEV. Biophysical experiments demonstrated direct binding between domain 1 of LDLRAD3 and the VEEV p62–E1 structural protein. Administration of a LDLRAD3-domain 1-Fc decoy molecule or gene editing of LDLRAD3 in mice abolished VEEV infection and pathogenesis. Cryo-EM studies revealed that LDLRAD3 engages VEEV E2–E1 in a mechanism analogous to that by which CHIKV engages MXRA8, albeit with a smaller footprint on the virion[68,69]. Both MXRA8 and LDLRAD3 recognize similar sites on the virion that encompass residues in both E2 (domains A and B) and E1 (domain II) proteins. Most recently, another CRISPR–Cas9 screen identified two additional LDL-scavenger receptors, VLDLR and ApoER2, that promote infection by SFV, EEEV and SINV[70]. Administration of a VLDLR-Fc decoy molecule prolonged survival in SFV-infected mice.

The identification of NRAMP2, MXRA8, LDLRAD3, VLDLR and ApoER2 as alphavirus host receptors may explain the broad and differential host tropism of alphaviruses. Structural studies are needed to determine whether NRAMP2, VLDLR and ApoER2 engage the E2–E1 proteins of SINV, SFV and EEEV in a manner similar to the engagement of MXRA8 and LDLRAD3 with other alphaviruses. Understanding the molecular and structural basis of the interaction between alphaviruses and host receptors could facilitate the identification of mAbs, receptor decoys or small-molecule inhibitors that prevent alphavirus entry, infection and pathogenesis.

Protective monoclonal antibodies

The use of mAb therapy to combat emerging viruses has recently been shown as a promising and feasible strategy against SARS-CoV-2 as both prophylaxis and therapy[71,72]. Modifications to the Fc region can extend the half-life of mAbs up to approximately 90 days[71,73]. Over the past five decades, antibody-mediated protection against alphavirus infection has been studied intensively. Passive transfer of purified polyclonal antibodies or mAbs with neutralizing activity confers protection against alphavirus challenge in animals. Non-neutralizing alphavirus mAbs can also confer protection in animals via Fc effector functions and engagement of cell-mediated immune mechanisms. In all cases, protective antibodies target the E3, E2 and E1 proteins on the alphavirus heterodimer.

In early studies, researchers infected mice with different alphaviruses and used B cell–myeloma fusion and hybridoma technology to generate mAbs against the structural proteins of SFV[74,75], SINV[76,77], RRV[78] and the encephalitic alphaviruses[79–81]. The most protective mAbs in

vivo recognized different epitopes on the alphavirus E2 or E1 protein. These seminal studies used competition binding assays to classify and identify mAbs as specific to alphavirus E2 or E1 proteins. Although these experiments provided valuable insight as to how antibody-mediated protection occurred, this Review highlights more recently described mouse and human mAbs (Table 1) against the alphavirus structural proteins, including many mAbs with comprehensive epitope mapping or structural characterization, extensive in vivo protection data, mechanism-of-action data and cross-reactivity among alphaviruses.

E3 antibodies

The E3 protein participates in transport, stabilization and maturation of the E2–E1 protein, and its dissociation from the virion upon release into the extracellular space enables priming of the E2–E1 spike protein for membrane fusion upon exposure to the acidic environment of the endosome[23,24,82]. Nonetheless, a cleaved form of E3 can remain bound to the virion of some alphaviruses and under specific culture conditions[15,24,25]. A panel of mAbs against the E3 protein were isolated from mice inoculated with a recombinant VEEV strain containing a mutation in the furin cleavage site that prevents furin processing and subsequent release of the E3 glycoprotein[83]. These mAbs neutralized infection by the mutant but not by the parental VEEV strain. Interestingly, among the six anti-E3 neutralizing mAbs, mAb 13D4 protected mice from a lethal challenge with the parental VEEV strain. The mechanism of neutralization and protection by anti-E3 mAbs warrants further study. Moreover, protective or neutralizing mAbs against the E3 protein of arthritogenic alphaviruses have not yet been reported.

E2 antibodies

Epitopes recognized by neutralizing human and mouse antibodies have been identified in all three domains of the E2 protein (Fig. 2a–c). Neutralizing mAbs against the E2 glycoprotein of arthritogenic alphaviruses (CHIKV[84–91], RRV[86,90,92,93], ONNV[86,90], BFV[86,87], MAYV[86,90,94], SINV and SFV[86,90]) and encephalitic alphaviruses (EEEV[95,96], WEEV[97] and VEEV[79,81,98,99]) have been isolated using classic hybridoma fusions, single-antigen-specific B cell sorting or phage display.

Domain A mAbs. The finding that some mAbs targeting alphavirus E2 domain A could neutralize infection was anticipated based on the hypothesis that this region engaged host cell receptors[27,100–103]. Although the amino acid sequence of domain A varies among alphaviruses (34–93% sequence identity), several studies have identified mAbs against different alphaviruses that bind in similar regions of domain A and share functional properties. Nonetheless, most neutralizing and protective domain A mAbs are type-specific and do not cross-react or cross-protect against heterologous alphaviruses.

Within domain A, both neutralizing and protective mAbs have been localized predominantly to residues 56–81 and, to a lesser extent, residues 99–121, indicating an exposed protective epitope (Fig. 2a). Here we highlight selected mAbs for which the binding epitopes and protective activity have been extensively evaluated. The mAb CHK-152 was isolated from CHIKV-infected mice after viral clearance and potently neutralized laboratory-adapted and clinical strains by inhibiting viral fusion[85]. Through neutralization escape selection[85], residue 59 in domain A was identified as a critical interaction residue, which was corroborated by cryo-EM studies[100]. Passive transfer of CHK-152 protected against CHIKV challenge in immunocompetent and immunocompromised mice[85] and reduced virus dissemination and tissue injury in rhesus macaques[104]. Human mAbs targeting similar epitopes in domain A of E2 have been identified from individuals with a previous history of arthritogenic alphavirus infection. Potently neutralizing mAbs isolated from individuals infected with CHIKV through either hybridoma fusion[87] or phage display (mAb IM-CKV063)[88] were mapped to domain A residues 55–81 and 99–121 through alanine-scanning mutagenesis and shown to protect mice against CHIKV pathogenesis[88]. In addition, functionally important human mAbs against RRV (mAbs RRV-92 and RRV-196) that engage residues 60–75 within domain A were isolated from individuals with a previous history of RRV infection[92]. Two RRV mAbs (RRV-92 and RRV-196) and IM-CKV063 protected mice against RRV and CHIKV infection, respectively.

Despite the sequence variation in domain A between arthritogenic and encephalitic alphaviruses, protective mAbs that target similar residues in encephalitic alphaviruses have been identified. Panels of mouse and human mAbs against domain A of the EEEV E2 glycoprotein have been isolated[95,96,105]. Through neutralization escape, mutagenesis and cryo-EM studies, three protective mouse mAbs (EEEV-18, EEEV-82 and EEEV-102) were mapped to residue 68 in domain A in addition to amino acids in domain B[19,95]. The human mAb EEEV-33 protected mice from lethal EEEV aerosol challenge and engaged domain A residues 34, 74 and 116–120 along with some contacts in domain B (residues 195–197)[96]. These mAbs inhibited early entry stages of EEEV infection. Neutralizing and protective VEEV mAbs have been mapped to similar residues in domain A by escape selection, alanine-scanning mutagenesis and structural analysis[98,106]. Protective mouse anti-VEEV mAbs (mVEEV-36 and mVEEV-68) bound an epitope in domain A spanning residues 56–64 (ref.[98]). These mAbs inhibited most stages of VEEV infection, including viral attachment to the LDLRAD3 receptor, fusion and egress, and protected mice against VEEV infection[98]. Collectively, the identification of mouse and human mAbs against domain A residues 56–81 establishes a protective epitope shared by both arthritogenic and encephalitic alphaviruses.

Domain A mAbs with poor neutralizing activity can also confer protection. The most protective non-neutralizing anti-MAYV mAbs recognized two linear epitopes in domain A (residues 57–61 and 159–163) (Fig. 2a), demonstrated high affinity to MAYV virions and prevented MAYV-induced musculoskeletal disease[107]. Protective activity against MAYV was mediated through monocyte-dependent Fc effector functions, as loss of protection occurred with monocyte depletion or introduction of mutations that abrogated engagement of Fc-γ receptors[108,109]. Similar findings were reported for a poorly neutralizing mAb, mVEEV-43, which protected mice against VEEV challenge, although the mechanism of action was not investigated[98]. Protection may be mediated by elimination of infected cells by antibody-dependent cellular phagocytosis or cytolysis, as alphavirus-infected cells display high levels of E2–E1 proteins on the cell surface[42,108]. An alternative protective mechanism, as demonstrated for SINV, may be non-cytolytic clearance of virus in which neutralizing domain A mAbs signal to decrease viral RNA synthesis and budding[110–112]. Interestingly, both non-neutralizing and neutralizing domain A mAbs against MAYV, CHIKV, RRV, EEEV and VEEV recognize proximal epitopes (for example, targeting residues 57–61). Although further analysis is required, differences in epitope recognition including the angle of mAb approach or tertiary and quaternary interactions could determine the neutralizing activity of mAbs that ostensibly bind to similar epitopes.

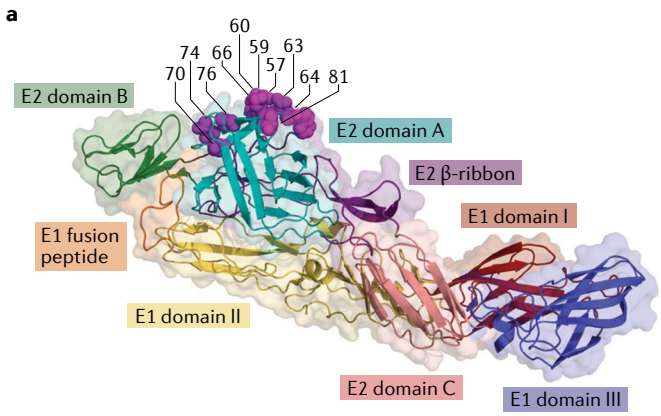
Domain B mAbs. Domain B is the most membrane-distal region of the alphavirus E2–E1 heterodimer[15,16,30] and another target of neutralizing and protective mAbs (Fig. 2b). Domain B is positioned above the

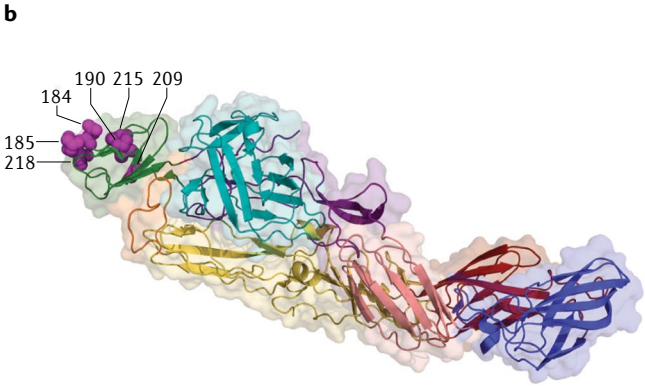
Table 1 | Summary of alphavirus monoclonal antibodies described in this Review

Domain	Alphavirus class	Antibody	Species	Key binding residues within specified domain[a]	Alphavirus reactivity	Protective efficacy (prophylactic)	Protective efficacy (therapeutic)
E2 domain A	Arthritogenic	CHK-152	Mouse	D59	CHIKV	>70% survival	30–70% survival
		IM-CKV063	Human	E24, G55, W64, K66, R80, I121	CHIKV	>70% survival	30–70% survival
		MAY-X Abs[b]	Mouse	S27-H29, T57-T61, G72-E77, S81-H86	MAYV	>70% survival	30–70% survival
		RRV-92	Human	K66, V75	RRV	30–70% survival	ND
		RRV-196	Human	G60, K66, Y69	RRV	ND	ND
	Encephalitic	EEEV-18	Mouse	M68	EEEV	>70% survival	30–70% survival
		EEEV-82	Mouse	M68, L81	EEEV	>70% survival	30–70% survival
		EEEV-102	Mouse	M68, L81	EEEV	ND	ND
		hEEEV-33	Human	E34, K74, G116, N118, H120	EEEV	>70% survival	<30% survival
		mVEEV-36	Mouse	D56, K62	VEEV	>70% survival	>70% survival
		mVEEV-68	Mouse	D56, K62, G63, R64, D94	VEEV	>70% survival	>70% survival
		mVEEV-43	Mouse	T49, I110	VEEV	>70% survival	30–70% survival
E2 domain B	Arthritogenic	CHK-265	Mouse	Q184, S185, I190, V197, Y199, G209, L210, T212, I217	CHIKV, ONNV, MAYV, RRV, BEBV, UNAV, SFV, GETV	>70% survival	ND
		DC2.M16	Human	G209, K215	CHIKV, MAYV	>1 $\log_{10}$-fold reduction in viral titres	ND
		DC2.M108	Human	G209, K215	CHIKV, MAYV, ONNV	ND	ND
		DC2.M357	Human	K189, N218	CHIKV, ONNV, MAYV, RRV, SFV	>1 $\log_{10}$-fold reduction in viral titres	ND
		MAY-117	Mouse	L181, S182, Q183, Q184, S185, G186, I190	MAYV, CHIKV, RRV, UNAV	30–70% survival	ND
		RRV-12	Human	Q183-N187, N218-K221, D223	CHIKV, ONNV, MAYV, RRV, GETV, SAGV	ND	30–70% survival
E2 domain C	Arthritogenic	RRV-210	Human	T283, D289	RRV	ND	ND
		RRV-221	Human	T283	RRV	ND	ND
		mVEEV-19	Mouse	N332	VEEV	ND	ND
		mVEEV-68	Mouse	N332	VEEV	>70% survival	30–70% survival
E1 domain II	Arthritogenic	CHK-166	Mouse	K61, G64	CHIKV	>70% survival	30–70% survival
		DC2.112	Human	W89, G91, N100	CHIKV, ONNV, MAYV, RRV, BEBV, UNAV, SINV, BBKV, OCKV, BCRV, EEEV, VEEV, WEEV	>70% survival	>70% survival
		DC2.315	Human	K52, V54, I55, K61, G83, V84, Y85, M88, Y93, F95, N100	CHIKV, MAYV, RRV, BEBV, UNAV, BCRV, EEEV, VEEV, WEEV	>70% survival	>70% survival
		IM-CKV057	Human	W89, N100	CHIKV, EEEV	ND	ND
		IM-CKV061	Human	W89, N100	CHIKV, EEEV	ND	ND
		IM-CKV062	Human	W89, N100	CHIKV, EEEV	ND	ND
		IM-CKV066	Human	G83, Y85, F87, D97	CHIKV	ND	ND
		IM-CKV067	Human	W89, N100	CHIKV, EEEV	ND	ND
	Encephalitic	EEEV-138	Human	F95, N100	CHIKV, EEEV, MADV, VEEV, WEEV	ND	30–70% survival
		EEEV-179	Human	Y93	CHIKV, EEEV, MADV, VEEV, WEEV	ND	>70% survival
		EEEV-346	Human	N100	CHIKV, EEEV, VEEV, WEEV	30–70% survival	30–70% survival
		1A4B-6	Mouse	C94, C96, N100	CHIKV, RRV, SFV, SINV, EEEV, VEEV, WEEV	>70% survival	ND

Abs, antibodies; BBKV, Babanki virus; BCRV, Buggy Creek virus; BEBV, Bebaru virus; CHIKV, chikungunya virus; EEEV, Eastern equine encephalitis virus; GETV, Getah virus; MADV, Madariaga virus; MAYV, Mayaro virus; ND, not determined; OCKV, Ockelbo virus; ONNV, O'nyong'nyong virus; RRV, Ross River virus; SAGV, Sagiyama virus; SFV, Semliki Forest virus; SINV, Sindbis virus; UNAV, Una virus; VEEV, Venezuelan equine encephalitis virus; WEEV, Western equine encephalitis virus. [a]Residue numbering is based on the autologous virus from which the monoclonal antibody was generated. [b]Multiple non-neutralizing anti-MAYV monoclonal antibodies[92].

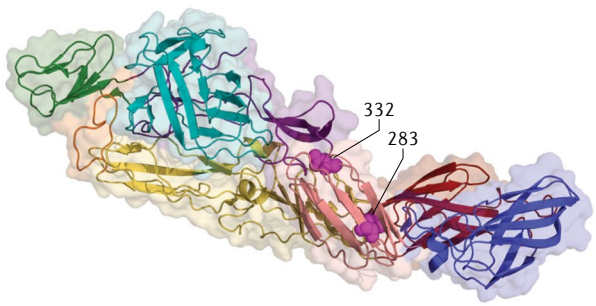
fusion loop and prevents premature fusion loop exposure. As with domain A, several studies have described mouse and human mAbs to domain B that protect against arthritogenic or encephalitic alphavirus infection. The relatively conserved sequence of domain B among arthritogenic alphaviruses (CHIKV versus ONNV: 86%)[86] correlates with the identification of cross-reactive and cross-protective mAbs that target two epitopes spanning residues 180–199 in the A–B strands and 209–223 in the C–C′ strands, respectively (Fig. 2b). Studies on mechanism of action revealed that these domain B mAbs inhibit multiple stages of viral infection, including virus attachment, fusion and egress.

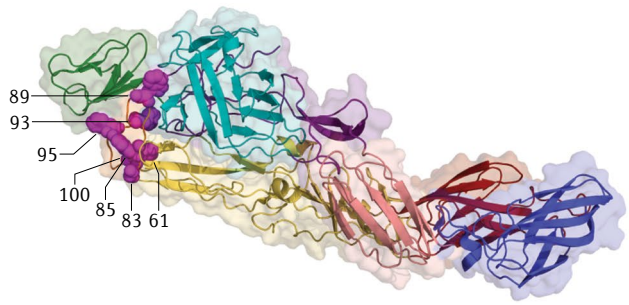




E2 domain A		
Arthritogenic		
CHIKV	Neutralizing:	CHK-152, IM-CKV063
MAYV	Non-neutralizing:	MAYV mAbs
RRV	Neutralizing:	RRV-92, RRV-196
Encephalitic		
EEEV	Neutralizing:	EEEV-18, EEEV-82, EEEV-102, hEEEV-33
VEEV	Neutralizing:	mVEEV-36, mVEEV-68
VEEV	Non-neutralizing:	mVEEV-43

E2 domain B		
Arthritogenic		
CHIKV	Neutralizing:	CHK-265, DC2.M357, DC2.M16, DC2.M108
MAYV	Neutralizing:	MAY-117
RRV	Neutralizing:	RRV-112





E2 domain C		
Arthritogenic		
RRV	Neutralizing:	RRV-210, RRV-221
Encephalitic		
VEEV	Neutralizing:	mVEEV-19, mVEEV-68

E1 domain II		
Arthritogenic		
CHIKV	Neutralizing:	CHK-166
CHIKV	Non-neutralizing:	DC2.112, DC2.315, IM-CKV057, IM-CKV061, IM-CKV062, IM-CKV066, IM-CKV067
Encephalitic		
EEEV	Non-neutralizing:	EEEV-138, EEEV-179, EEEV-307, EEEV-354, EEEV-342, EEEV-346, EEEV-368, 1A4B-6

Fig. 2 | Neutralizing and non-neutralizing monoclonal antibody epitopes on the alphavirus p62–E1 heterotrimer. The key amino acids of alphavirus monoclonal antibodies (mAbs) described in this Review are highlighted on the chikungunya virus (CHIKV) E2–E1 heterodimer (Protein Data Bank ID: 6NK5). Each E2–E1 panel highlights the amino acids of protective epitopes (magenta spheres) from each mAb class (E2 domain A (part **a**), E2 domain B (part **b**), E2 domain C (part **c**) and E1 domain II (part **d**)) that are shared by two or more mAbs. CHIKV amino acid numbering is used. EEEV, Eastern equine encephalitis virus; hEEEV, human EEEV; MAYV, Mayaro virus; mVEEV, mouse VEEV; RRV, Ross River virus; VEEV, Venezuelan equine encephalitis virus.

The mouse mAb CHK-265, which was isolated from mice infected with CHIKV[85], neutralized multiple arthritogenic alphaviruses including CHIKV, MAYV, RRV, ONNV and SFV[86,113]. Mutagenesis and cryo-EM experiments revealed that CHK-265 recognizes residues in both the A–B strands (residues 184, 185, 190, 197 and 199) and the C–C′ strands (residues 209, 210, 211 and 217). RRV-12, which was isolated from an RRV-immune individual, potently neutralized multiple arthritogenic alphaviruses and bound to RRV domain B residues 183–187, 218–221 and 223, analogous to CHK-265 (ref.[93]). Both CHK-265 and RRV-12 inhibited multiple steps of the viral replication cycle and protected mice against infection and disease caused by CHIKV, MAYV and RRV. DC2.M357, which was isolated by single-B cell sorting from an individual with a history of CHIKV infection, neutralized infection by CHIKV, MAYV, ONNV, RRV and SFV[90]. Neutralization escape selection studies with DC2.M357 showed that binding of this mAb depends on residues 189 and 218 in domain B, which are proximal to the CHK-265 and RRV-12 epitopes. Cross-reactive neutralizing mAbs have also been isolated from mice inoculated with MAYV[94]. The protective mAb MAY-117 shares a virtually identical epitope with CHK-265 and RRV-12, as mutagenesis studies identified domain B residues 184, 185, 190 and 210 as critical for binding. Collectively, the overlapping binding epitopes, cross-reactivity and therapeutic studies indicate that some domain B-binding mAbs (for example, CHK-265, RRV-12, DC2.M357 and MAY-117) share a conserved, broadly neutralizing and protective epitope.

Cross-reactive mAbs with more limited breadth against arthritogenic alphaviruses that map to a second epitope in domain B have been identified through single-B cell sorting[89,90]. Virus neutralization escape studies identified domain B residues 209 and 215 as critical for neutralization by mAbs DC2.M16 and DC2.M108. These residues are separate from the binding epitope of CHK-265, RRV-12 and DC2.M357. Although many arthritogenic or encephalitic type-specific domain B mAbs have been isolated[85–87,92,95,96,98,99,114,115], certain molecular determinants, such as recognition of residues 184 and 185, seem to be required for cross-reactivity. Although mAbs that are broadly reactive to this epitope have been elicited to arthritogenic alphaviruses in both mice and humans, such mAbs have not been described for encephalitic alphaviruses, possibly owing to the greater sequence divergence in domain B between EEEV, VEEV and WEEV (31–46%)[15].

Domain C mAbs. Domain C is located proximal to the virus membrane and is much less accessible for mAbs to bind on the prefusion alphavirus trimer[15,16]. However, mAbs to RRV[92] and VEEV[98] domain C have been identified that block alphavirus engagement with host receptors, although these mAbs have additional contacts in domains A and/or B (Fig. 2c). In this section, we briefly highlight neutralizing domain C mAbs.

From individuals with previous RRV infection, two neutralizing mAbs, RRV-210 and RRV-221, were shown through alanine-scanning mutagenesis to recognize residue 283 in domain C along with residues in domains A and B[92]. These mAbs blocked MXRA8 receptor binding to RRV, thereby demonstrating a possible neutralization mechanism. Two neutralizing mAbs from mice inoculated with VEEV, mVEEV-19 and mVEEV-68, which also blocked LDLRAD3 receptor binding, bound residue 332 in domain C[98]. Analogous to the RRV mAbs, the VEEV mAbs also engaged residues in domain A. mVEEV-68 conferred protection against VEEV in mice, suggesting that domain C might be a protective mAb epitope. However, as these mAbs recognize residues in multiple domains, how much of this protection originates from domain C remains to be determined.

E1 antibodies

Although the E1 protein is not exposed to the same extent as the E2 protein, alphavirus-specific and cross-reactive E1 mAbs to SINV and encephalitic alphaviruses have been reported[80,116–120]. The E1 protein comprises three domains (I, II and II) and is situated beneath the E2 protein (Fig. 1). Domain I is located centrally in the E1 protein between domains II and III and is relatively inaccessible on the mature virion[15]. Domain III is located proximal to the viral membrane. To date, no neutralizing or protective mAbs to domains I or III have been identified. Within domain II is the hydrophobic fusion loop responsible for membrane fusion, and mAbs to this domain have been described. Although a majority of E1 mAbs have poor neutralizing activity in vitro, some protect in vivo[85,119]. In contrast to E2 mAbs, which protect primarily through neutralization, mAbs binding to epitopes proximal and within the fusion loop in domain II of E1 (Fig. 2d) protect by inhibiting viral morphogenesis and egress or through Fc-mediated effector mechanisms[121–123].

The mAb CHK-166 was isolated from mice infected with CHIKV and mapped through neutralization escape to residue 61 in domain II of E1, which is proximal to the fusion loop[85]. Although CHK-166 exhibited moderate neutralizing activity in vitro, monovalent or combination therapy with CHK-152 protected mice against CHIKV-induced mortality principally through a mechanism dependent on monocyte-mediated Fc effector functions[85,124]. Other studies identified poorly neutralizing mAbs against residues in the E1 fusion loop (for example, F95 and N100) that were broadly cross-reactive to both arthritogenic and encephalitic alphaviruses and protective against multiple alphaviruses in mice[121,122]. These epitopes were not accessible on the intact virion, which explains the poor neutralizing activity of these mAbs. The EEEV mouse mAb 1A4B-6, which cross-reacts with multiple alphaviruses including CHIKV, RRV, SINV, SFV, EEEV, VEEV and WEEV, was shown to protect mice against a lethal VEEV challenge[116,123]. As with previously reported protective E1 mAbs[121,122], subsequent alanine-scanning mutagenesis experiments identified N100 as a critical E1 residue for 1A4B-6 binding[123].

Other studies have identified mAbs to similar residues in domain II, although the in vivo efficacy or protective mechanisms were not determined. From a phage display library constructed from the peripheral blood of individuals immune to CHIKV, five E1 mAbs were found to recognize similar key residues (for example, residue N100) in the fusion loop through alanine-scanning mutagenesis[88]. From mice immunized with CHIKV 6K–E1 protein, a subset of mAbs with poor neutralizing activity were established as cross-reactive against both arthritogenic and encephalitic alphaviruses, although protection and key binding residues were not evaluated[125].

The isolation of human and mouse mAbs to the E1 fusion loop from both natural infection and immunization suggests that the alphavirus fusion loop is an immunodominant epitope. The E1 residue N100, at the distal end of the fusion loop, is highly conserved among all alphaviruses[15,121] and is a critical binding residue for all broadly protective E1 mAbs (Fig. 2d). This class of E1 mAbs holds potential as a pan-alphavirus immunotherapy and presents a new target towards the development of a pan-alphavirus immunogen.

Development of antibody-based therapies

To date, no alphavirus mAb has been licensed for use in humans. However, mAb therapy has been shown to have promise in the prevention of virus infection[126] as demonstrated with respiratory syncytial virus[127] and more recently with SARS-CoV-2 (refs.[128,129]). Passive immunization

of mice and nonhuman primates (NHPs)[130] with neutralizing mAbs has been demonstrated to confer protection against alphavirus infection. In this section, we highlight the preclinical studies demonstrating protective efficacy of alphavirus mAbs beyond mouse models and one that utilized a novel delivery platform to control alphavirus infection.

Both mAb monotherapy and combination therapy have been shown to have therapeutic efficacy against alphavirus infection in NHP models. Administration of the E2 mAb 4N12 (ref.[87]) to rhesus macaques reduced CHIKV-induced disease and acute inflammation[130]. In an NHP study with VEEV, two anti-E2 mAbs, 1A3B-7 and 1A4A-1, protected animals against severe VEEV disease and reduced viraemia as monotherapy[131]. Despite the protective efficacy of these mAbs, viral sequencing of serum from NHPs revealed escape mutations in their domain B epitope within 4 days of infection. To overcome rapid viral escape from mAb therapy, combination therapies have been evaluated. Therapeutic administration of mAbs CHK-152 and CHK-166 protected rhesus macaques from CHIKV infection and dissemination[104]. Although previous studies identified virus escape mutants against CHK-152 or CHK-166 in cell culture or in mice when used as monotherapy, escape mutations were not identified in the rhesus macaques given combination therapy[85,104]. Thus, combination therapy against two separate mAb epitopes may limit the emergence of viral escape mutations and resistance.

Although the studies described above have utilized passive transfer of mAbs in animals, limitations exist including the medical complexity of delivery and high cost of treatment. To overcome these limitations, a recent study evaluated whether lipid nanoparticles (LNPs) containing mRNA encoding a potently neutralizing CHIKV mAb could protect against infection and disease[132]. Administration of mAb CHKV-24 protected mice from CHIKV-induced disease and abrogated viraemia. When evaluated in an NHP model, protective levels of mAb CHKV-24 were achieved upon LNP administration. In addition, CHKV-24 mRNA-LNPs have been evaluated in clinical trials and produced no serious adverse effects (NCT03829384)[133]. Given the safety and recent success with mRNA-based vaccines against SARS-CoV-2 (ref.[134]), the development of mRNA-encoded mAbs may hold promise for limiting alphavirus infection and disease.

Table 2 | Alphavirus vaccine candidates in advanced clinical development

Type of vaccine	Vaccine candidate	Clinical trial sponsor	Clinical trial identifier
Live-attenuated	VLA1553 (CHIKV) VEE 3526 (VEEV) VEE TC-83 (VEEV)	Valneva DynPort Vaccine USAMRIID	NCT04546724 NCT00109304 NCT00582504
Inactivated	BBV87 (CHIKV) TSI-GSD 104 (EEEV) VEE C-84 (VEEV) TSI-GSD 210 (WEEV)	Bharat Biotech USAMRIID USAMRIID USAMRIID	NCT04566484 NCT00584805 NCT00582088 NCT02466750, NCT01159561
Virus-like particle	VRC-CHKVLP059 (CHIKV)	NIH/NIAID	NCT01489358, NCT02562482
	PXVX0317 (CHIKV)	Emergent BioSolutions	NCT03483961, NCT05072080
	VRC-WEVVLP073 (EEEV/VEEV/WEEV)	NIH/NIAID	NCT03879603
Viral vector	MV-CHIK (CHIKV)	Themis Bioscience	NCT03101111, NCT02861586, NCT03807843
	ChAdOx1 Chik (CHIKV)	University of Oxford	NCT03590392
mRNA-based	mRNA-1944 (CHIKV)	Moderna	NCT03829384

CHIKV, chikungunya virus; EEEV, Eastern equine encephalitis virus; NIAID, National Institute of Allergy and Infectious Diseases; USAMRIID, United States Army Medical Research Institute of Infectious Diseases; VEEV, Venezuelan equine encephalitis virus; WEEV, Western equine encephalitis virus.

Alphavirus vaccine approaches

Although alphavirus vaccines are not currently approved for humans, many candidates have been evaluated in animals and advanced to clinical-phase testing. Various approaches have been used to generate live-attenuated viral vaccines, including the introduction of attenuating mutations or deletions in structural and nonstructural proteins or the ablation of the p62 furin cleavage site[135–145]. Inactivated virus vaccines have been generated by chemical treatment of infectious virions, for example, with formalin[137,146,147]. Virus-like particle (VLP) vaccines, which are composed of only the structural proteins, recapitulate the structural features of the virion but are not infectious[148–150]. Adenoviral and measles-based vectors have been used as a delivery platform for alphavirus protein immunogens[145,151–154]. These viral vectors often induce more potent CD8 T cell responses[155]. Several alphavirus candidates prevent alphavirus infection and disease in animals and have been advanced into clinical trials, which we review here (Table 2).

Given the epidemic potential of CHIKV, most clinical trials have focused on vaccines against this emerging alphavirus. Current vaccines against CHIKV that are under evaluation include recombinant VLPs composed of the structural proteins C–E3–E2–6K–E1 (NCT03483961, NCT05072080, NCT02562482 and NCT01489358)[156,157], measles (NCT03101111, NCT02861586 and NCT03807843)[158] and adenoviral (NCT03590392)[159] vectors that express the C–E3–E2–6K–E1 structural proteins, and live-attenuated viruses (NCT04546724 and NCT04566484). Generally, these vaccines have been shown as safe and immunogenic in early clinical trials. One of the most advanced candidates is the live-attenuated VLA1553 CHIKV vaccine, which has a large deletion in the nsP3 replicase region[145]. In a recently completed phase III trial, VLA1553 was shown to have good safety and tolerability profiles and to elicit high levels of neutralizing antibody in serum (NCT04546724). Because encephalitic alphaviruses pose a substantial threat due to high mortality, neurological sequelae and possible weaponization, clinical trials to evaluate encephalitic alphavirus vaccines have begun. Live-attenuated vaccine trials against EEEV (NCT02654509 and NCT00584805), VEEV (NCT00109304, NCT00582504 and NCT00582088) and WEEV (NCT02466750 and NCT01159561) have been initiated, although there are concerns of reactogenicity and limited immunogenicity[160,161]. To address these limitations, replication-incompetent trivalent VLPs composed of the EEEV, VEEV and WEEV structural proteins were generated based on a strategy utilized for the CHIKV VLP-based vaccine[148,150]. Administration of the trivalent vaccine to NHPs conferred complete protection against all three encephalitic alphaviruses, and this vaccine is currently under evaluation in a phase I clinical trial (NCT03879603).

Concluding remarks

The identification of mAbs that engage protective epitopes on arthritogenic and encephalitic alphaviruses provides an opportunity to understand humoral immune responses during infection or immunization, develop mAb therapeutic strategies and, more importantly, guide iterative vaccine design. There are numerous challenges with vaccine development, but the understanding of immunodominant and protective B cell epitopes during alphavirus infection is a major component

of advancing vaccine development. In theory, such information can be used to focus the induction of neutralizing mAbs on protective alphavirus epitopes through reverse vaccinology[162]. Although this Review has focused on mAb neutralization as a primary protective mechanism, other immune correlates should be considered during alphavirus vaccine design including T cell responses and cell-mediated immunity. The most advanced alphavirus vaccine clinical trials to date (NCT04546724 and NCT03483961) have focused on analysing neutralizing antibody responses, although going forward it will be important to profile other responses when evaluating immune correlates of protection. The intensive study of antibody protection, receptor biology, antigenicity and vaccine development has enabled the development and new strategies to counteract alphavirus infection. Such approaches may provide a pathway for the rapid development of countermeasures against alphavirus infection that limit future morbidity, mortality and epidemic transmission.

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Additional information
Correspondence should be addressed to Michael S. Diamond.

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