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Review article

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Generating prophylactic immunity against arboviruses in vertebrates and invertebrates

Daniel J. Rawle[1], Leon E. Hugo[1], Abigail L. Cox[1], Gregor J. Devine[1,2] & Andreas Suhrbier[1,2]

Abstract

The World Health Organization recently declared a global initiative to control arboviral diseases. These are mainly caused by pathogenic flaviviruses (such as dengue, yellow fever and Zika viruses) and alphaviruses (such as chikungunya and Venezuelan equine encephalitis viruses). Vaccines represent key interventions for these viruses, with licensed human and/or veterinary vaccines being available for several members of both genera. However, a hurdle for the licensing of new vaccines is the epidemic nature of many arboviruses, which presents logistical challenges for phase III efficacy trials. Furthermore, our ability to predict or measure the post-vaccination immune responses that are sufficient for subclinical outcomes post-infection is limited. Given that arboviruses are also subject to control by the immune system of their insect vectors, several approaches are now emerging that aim to augment antiviral immunity in mosquitoes, including *Wolbachia* infection, transgenic mosquitoes, insect-specific viruses and paratransgenesis. In this Review, we discuss recent advances, current challenges and future prospects in exploiting both vertebrate and invertebrate immune systems for the control of flaviviral and alphaviral diseases.

Sections

[1]QIMR Berghofer Medical Research Institute, Brisbane, Queensland, Australia. [2]GVN Centre of Excellence, Australian Infectious Disease Research Centre, Brisbane, Queensland, Australia. e-mail: Andreas.Suhrbier@qimrberghofer.edu.au

Introduction

In 2022, the World Health Organization (WHO) launched the Global Arbovirus Initiative to combat diseases caused by viruses that are transmitted by arthropod (insect) vectors[1,2]. Pathogenic flaviviruses and alphaviruses, which are mainly transmitted by mosquitoes (and occasionally by other arthropod vectors such as ticks), present the major arboviral burden to human and animal health globally. These include flaviviruses such as dengue virus (DENV), yellow fever virus (YFV) and Zika virus (ZIKV), and alphaviruses such as chikungunya virus (CHIKV) and Venezuelan equine encephalitis virus (VEEV). A summary of the viraemia, disease course, diagnosis and treatment of these viruses, as a broad overview of the subject matter in this Review, is provided in Fig. 1 and Supplementary Box 1. This Review does not cover arboviruses in the order *Bunyavirales* or the family *Reoviridae*.

The global cost of human diseases transmitted by the mosquito *Aedes aegypti*, which is the primary vector of urban arboviruses, was estimated to be at least US$87.3 billion in 2022, mainly associated with DENV infection[3]. Furthermore, the expanding global distribution of *Aedes albopictus*, a vector for, among others, ZIKV, CHIKV and DENV, has presented new opportunities for arboviral outbreaks in humans[4,5]. Flaviviruses and alphaviruses that cause economic loss in agricultural and veterinary settings include West Nile virus (WNV; a pathogen of humans, horses and birds)[6,7], Japanese encephalitis virus (JEV; a pathogen of humans, horses and pigs)[8,9], VEEV (a pathogen of humans and horses)[10,11], Getah virus (a pathogen of pigs and horses)[12] and Salmon pancreas disease virus (a pathogen of farmed salmon)[13].

A range of licensed flavivirus and alphavirus vaccines are currently available, including the recent approval of the CHIKV vaccine IXCHIQ (VLA1553) by the US Food and Drug Administration (FDA)[14] (Table 1 and Box 1). In addition, several vaccines have shown success in late-phase clinical trials, reflecting the desire to develop better human vaccines for CHIKV, ZIKV, WNV and others (Supplementary Table 1). Better human vaccines for the equine encephalitis viruses are also in early-phase clinical development[11], the importance of which is illustrated by a fatal case of Western equine encephalitis virus recently reported in Argentina, the first human case in more than two decades[15]. New YFV vaccines are in preclinical development[16]; these should be safer than the current live-attenuated vaccines (Table 1) for elderly, pregnant and immunosuppressed individuals[17] and not reliant for their manufacture on the sometimes limited global supply of specific-pathogen-free eggs.

The currently licensed vaccines often target diseases — for example, those caused by DENV, YFV, tick-borne encephalitis virus (TBEV) and Salmon pancreas disease virus — that have reasonably predictable reoccurrences within specific geographical regions and with sufficient case numbers to allow for phase III efficacy trials (human) or field trials (animal). For more epidemic viruses, such as CHIKV, ZIKV and VEEV, a major hurdle for such phase III vaccine trials (and thus approval and licensing) is that outbreaks are difficult to predict with respect to location, timing and case numbers. The high cost, regulatory processes and infrastructure associated with phase III trials are challenging for the rapid deployment of trials in outbreak areas, particularly in resource-poor settings. Even large outbreaks of disease, such as the CHIKV outbreak in Reunion Island[5] and the ZIKV outbreak in Brazil[18], can be over within approximately a year owing to the development of immunity in the population and the deployment of public health interventions. In such scenarios, in addition to the limited time frame to set up a trial, health-care systems may already be stretched and may have difficulties accommodating a large-scale phase III trial. To reduce the need for such trials, potential alternative or complementary routes to vaccine approval and licensing are now available (as exemplified by the recent approval of IXCHIQ; Box 1). Future approvals would be facilitated by a deeper understanding of the vaccine-induced immune responses that provide protection from disease (Box 2) as well as more consistent and compelling assays to measure them.

In addition to vaccine development for vertebrates, several strategies are emerging to suppress the transmission of pathogenic flaviviruses and alphaviruses by mosquitoes by replacing susceptible mosquito populations with populations that are refractory to infection. Existing strategies aim to control mosquito numbers, primarily through the use of insecticides, but insecticide resistance is now widespread in key mosquito populations around the world. In addition, getting insecticides to where and when they are needed in metropolitan areas can present considerable challenges. Thus, the impact of insecticide use on arboviral disease outbreaks can often be limited and additional approaches are warranted[19]. Transmission-blocking strategies involve prophylactic stimulation of antiviral immune defences in mosquitoes or artificially providing mosquitoes with antiviral immunity. The most advanced of these strategies, the use of *Wolbachia*-infected mosquitoes, is soon to receive preapproval by the WHO[20], providing a 'first-in-class' tool for the mitigation of arboviral transmission via the release of modified mosquitoes[21].

Here, we describe strategies for the generation of prophylactic immunity against flaviviruses and alphaviruses in both vertebrates and invertebrates. For vertebrates, we describe current vaccines and vaccines in late-stage development, the rationale behind the choice of immunogens, the types of immune response sufficient for vaccine-mediated protection, and the challenges for human efficacy trials for new vaccines. For invertebrates, we describe four main strategies — *Wolbachia* infection, transgenic mosquitoes, insect-specific viruses (ISVs) and paratransgenesis — to generate mosquito populations that are resistant to infection by and transmission of key arboviral pathogens through the promotion of antiviral immunity within the mosquito.

Prophylactic immunity in vertebrates

Vaccines remain the most cost-effective interventions for many flaviviral and alphaviral diseases[1,22], with several vaccines licenced for use in humans and animals in various parts of the world (Table 1). A range of new vaccines are also in advanced stages of development (Supplementary Table 1).

Structural considerations for vaccine immunogens

A common feature of many currently licensed flavivirus and alphavirus vaccines, as well as many in late-phase trials, is that whole-virus or virion particles are used, delivered either as live-attenuated or inactivated viruses or as virus-like particles (VLPs) (Table 1 and Supplementary Table 1). During viral replication, structural polyproteins are generated: capsid–pre-membrane–envelope (C–prM–E) for flaviviruses[23] and C–E3–E2–6K–E1 for alphaviruses[24]. After cleavage by a series of proteases, these polyproteins self-assemble to generate the virion particles, comprised of 90 E-protein homodimers for flaviviruses[25] and 80 trimeric E1–E2 heterodimers for alphaviruses[24]. This is a robust process that occurs in both vertebrate and invertebrate cells, both in vivo and in vitro, and provides the basis for the manufacture of VLP-based vaccines[26–29]. The targets of most neutralizing antibodies (Box 2) are the E-protein homodimers of flaviviruses and the trimeric E1–E2 heterodimers of alphaviruses, which are arrayed on the virion surface in specific pre-fusion conformations (Fig. 2). Therefore, viral

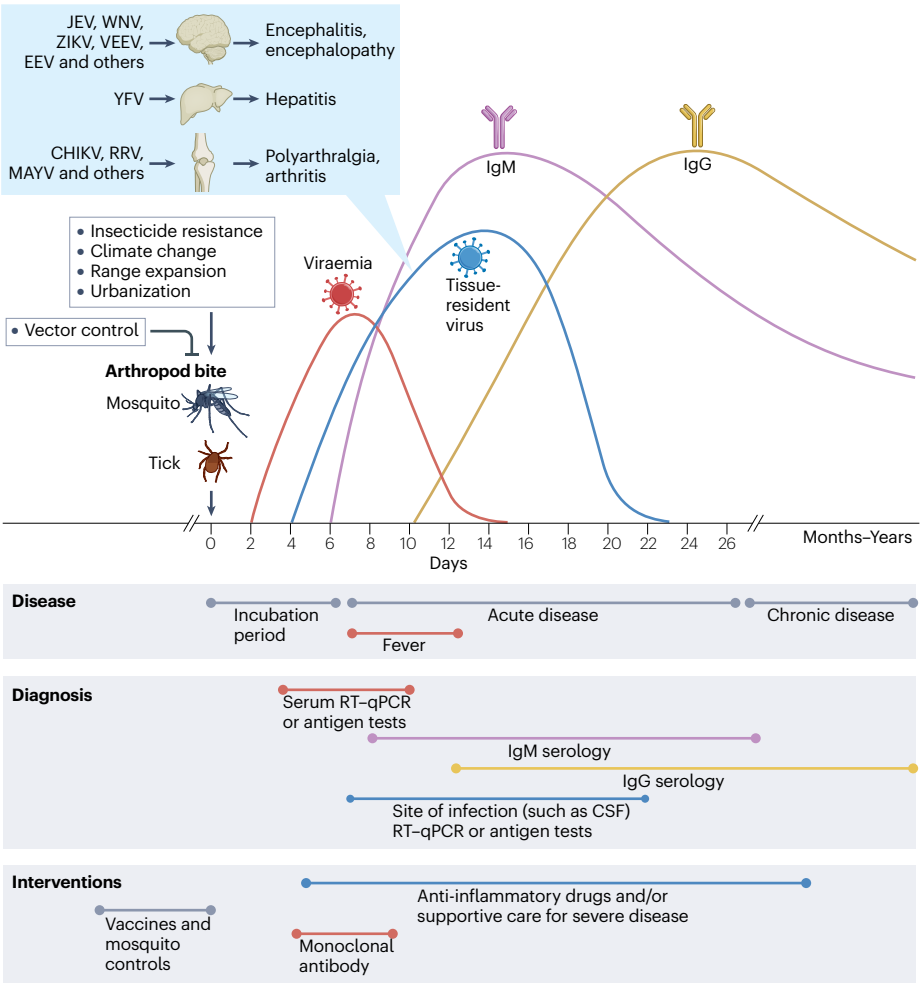


Fig. 1 | Flavivirus and alphavirus infections: viraemia, disease course, diagnosis and treatment. The primary arthropod vector for transmission of flaviviral and alphaviral diseases is the mosquito. A range of factors influence mosquito density (such as climate, urbanization and control measures such as insecticides) and disease transmission (such as biting rates on humans and their competence as disease vectors). Reducing virus transmission by mosquitoes is largely achieved by reducing mosquito numbers (vector control measures) but new strategies are emerging that seek to modify mosquitoes to make them less able to transmit viruses. Vaccination is a key intervention for flaviviruses and alphaviruses. In humans naive to these viruses, a bite from an infected arthropod (generally a mosquito or, in some cases, a tick) results in viraemia, the magnitude and duration of which can vary widely among individuals and between different viruses. In symptomatic cases, fever onset typically aligns with the peak viraemia and usually lasts for several days to a week. Serum reverse transcription quantitative PCR (RT–qPCR) or antigen tests thus have a limited period in which viral RNA or protein can be detected after symptom onset. Virus can also enter specific organs and tissues — such as the brain in the case of Japanese encephalitis virus (JEV), West Nile virus (WNV), Zika virus (ZIKV), Venezuelan equine encephalitis virus (VEEV) and equine encephalitis virus (EEV) (causing encephalitis), the liver in the case of yellow fever virus (YFV) (causing hepatitis), and the joints in the case of chikungunya virus (CHIKV), Ross River virus (RRV) and Mayaro virus (MAYV) (causing polyarthralgia and arthritis) — but these are usually not accessible for diagnostic purposes, although cerebrospinal fluid (CSF) can be tested for RNA or antigen from encephalitic viruses. IgM and IgG serology remain the main methods for diagnosis and often require two consecutive tests for confirmation[99]. The window of opportunity for potential monoclonal antibody therapies is narrow, requiring early RT–PCR diagnosis and prompt administration. Presently, anti-inflammatory drugs and/or supportive care for severe disease are the only standard treatment modalities (Supplementary Box 1).

immunogens with authentic pre-fusion tertiary and quaternary structures (similar to those found in infectious virion particles) are likely to be important for generating effective polyclonal neutralizing antibody responses[30–34]. The sites of antigen binding for neutralizing antibodies can, for example, involve two neighbouring E proteins[35,36] or two neighbouring trimeric E1–E2 heterodimers[37]. High-avidity binding of both Fab arms of an antibody can be envisaged[36,38] and may involve some Fab angle flexibility[39] and/or rotation (up to 180°)[40] (Fig. 2). Of note, IgG3, which has the greatest Fab orientation flexibility of all the human IgG subclasses[41], can dominate the neutralizing antibody responses to CHIKV[42] and ZIKV[43]. Distortion of the virion envelope conformation may also be involved in antibody recognition[38,44], with flaviviruses being known to 'breathe' (moving from pre-fusion to post-fusion conformations and back) and thereby expose epitopes that are inaccessible in the smooth pre-fusion structures[45]. Obtaining cryoelectron microscopy structures of high-avidity virus–IgG interactions is complicated by the

Table 1 | Licensed and approved flavivirus and alphavirus vaccines

Virus	Vaccine (manufacturer)	Construct type	Target population	Approval status (year of first approval)
Flavivirus vaccines				
Dengue virus (DENV serotypes 1–4)	Dengvaxia (Sanofi Pasteur)	Quadravalent, live-attenuated, YFV-17D backbone, Vero cell derived	Human; 9–45 years, with previous DENV infection	Licensed (2015)
	Qdenga (Takeda)	Quadravalent, live-attenuated, DENV2 backbone, Vero cell derived	Human; >4 years	Licensed (2022)
Yellow fever virus (YFV)	YF-VAX (Sanofi Pasteur; Stamaril)	Live-attenuated, YFV-17D, produced in chicken embryos	Human; >9 months	Licensed (1986)
	17DD-YFV Vaccine (Bio-Manguinhos/ Fiocruz)			WHO-prequalified manufacture since 2000
Japanese encephalitis virus (JEV)	IMOJEV (Sanofi Pasteur)	Live-attenuated, YFV-17D backbone, Vero cell derived	Human; >9 months	Licensed (2010)
	SA14-14-2 (Chengdu Institute of Biological Products)		Human; >1 year	Licensed (1988)
	IXIARO/JESPECT (Valneva)	Inactivated, alum adjuvant	Human; >2 months	Licensed (2009)
Tick-borne encephalitis virus (TBEV)	FSME-Immun or TicoVac (Pfizer)	Inactivated, alum adjuvant	Human; >16 years; junior formulation >1 year	Licensed (1976)
	Encepur (Bavarian Nordic)		Human; >12 years	Licensed (1992)
West Nile virus (WNV)	PreveNile (Intervet)	Live-attenuated, YFV-17D backbone, Vero cell derived	Horses	Licensed (2006)
	INNOVATOR (Zoetis)	Inactivated, MetaStim adjuvant		Licensed (2003)
	RECOMBITEK (Merial)	Recombinant canarypox		Licensed (2004)
Alphavirus vaccines				
Chikungunya virus (CHIKV)	IXCHIQ/VLA1553 (Valneva)	Live-attenuated, CHIKV LR2006-OPY1 with 61-amino-acid deletion in non-structural protein 3	Human; >18 years	Approved by FDA under the Accelerated Approval pathway (2023)
Salmon pancreas disease virus (SPDV)	Clynav (Elanco Animal Health)	DNA vaccine	Atlantic salmon (Europe)	Licensed (2017)
	AquaVac PD3 (MSD Animal Health)	Trivalent, including inactivated SPDV, light liquid paraffin adjuvant		Licensed (1983)
Venezuelan equine encephalitis virus (VEEV)	Equivac TC-83 (Productora Nacional de Biológicos Veterinarios)	Live-attenuated	Horses (Mexico)	FDA emergency use authorization for US Army (1963)
Eastern and Western equine encephalitis viruses (EEEV and WEEV), WNV+VEEV	Core EQ INNOVATOR+V (Zoetis)	Six-valent, inactivated, MetaStim adjuvant	Horses (USA)	Licensed (2018)
JEV+Getah virus (GETV)	JEV/GETV combined vaccine (Nisseiken)	Inactivated	Horses (Japan)	Used since 1979

FDA, Food and Drug Administration; WHO, World Health Organization.

high mobility imparted by the antibody hinge region and the propensity for virus–antibody complexes to aggregate[36]. However, obtaining more such structures for antibodies that are known to be highly neutralizing would help to refine our understanding of the structural requirements for optimal induction of protective antibody responses.

Vaccine modalities and technologies

Live-attenuated vaccines. Most flavivirus and alphavirus vaccines are either live-attenuated or inactivated whole-virus vaccines (Table 1 and Supplementary Table 1). The potential for undesirable dissemination as a possible risk of live-attenuated vaccines was recently highlighted by transmission of the live-attenuated YFV-17D vaccine through blood transfusion and organ transplantation[46]. For live-attenuated arbovirus vaccines, uptake and transmission by mosquitoes present additional potential risks such as recombination between the vaccine virus and circulating viruses[47,48], mutation of the vaccine virus resulting in reversion to increased virulence[48], and/or acquisition of virulence by the vaccine virus for off-target species[49]. Transmission to mosquitoes was seen for the live-attenuated TC-83 vaccine given to horses during a VEEV outbreak in the USA in the 1970s[10]. Although there were no known consequences of this transmission, new live-attenuated arboviral vaccines will need to demonstrate, to the satisfaction of regulators, an inability to be transmitted by relevant arthropod vectors. For example, the recently licensed live-attenuated Qdenga vaccine for DENV is reported as unlikely to be transmitted by *Ae. albopictus*[50], and YFV-17D has been shown to be incapable of transmission by *Ae. aegypti*[51]. Determinants of mosquito transmissibility are emerging[52], and such knowledge may assist in the design of future live-attenuated vaccines that cannot be transmitted.

Inactivated whole-virus vaccines. Inactivated whole-virus vaccines cannot be disseminated as infectious entities but such vaccines generally require an adjuvant for efficacy as they lack the self-adjuvanting properties inherent to live-attenuated vaccines[53,54] and must also be adequately inactivated. Most current inactivated whole-virus and VLP-based vaccines use aluminium-based adjuvants (simplified herein to alum) to boost immunogenicity[55] (Table 1 and Supplementary Table 1). However, several other adjuvants are now approved for use in humans (such as MetaStim, a squalene-based emulsion); therefore, choices beyond alum are increasingly available[56,57]. Formaldehyde is currently used for the inactivation of licensed flavivirus and alphavirus vaccines (Table 1), with incomplete formaldehyde inactivation of past VEEV vaccines believed to be the cause of sporadic outbreaks of VEEV in the Americas from 1938 to 1973 (ref. 58). Formaldehyde inactivation can reduce immunogenicity by irreversibly modifying lysine and (to a lesser extent) tryptophan residues, which leads to the loss of certain epitopes and potentially alters virion structure (discussed in ref. 59). Other, less damaging inactivation methodologies, such as ultraviolet or gamma irradiation or psoralen treatment, have been slow to reach the market for various reasons, including staff safety during manufacture, cost, scale-up issues and/or non-linear inactivation kinetics[60,61].

Chimeric ISV vaccines. Flaviviruses and alphaviruses that infect mosquitoes but do not infect vertebrates represent another approach for the development of whole-virus vaccines. Some of these ISVs are remarkably tolerant of substitution of their structural polyproteins with those of pathogenic flaviviruses and alphaviruses. This enables the generation of chimeric viruses that contain the genes encoding prM–E or C–E3–E2–6K–E1 vaccine antigens joined to the non-structural protein genes of the ISVs. Such chimeric viruses can be propagated in mosquito cell lines but are incapable of generating progeny in vertebrates, and can thus be used as intrinsically inactive, whole-virus vaccines. Prominent examples of ISVs that can be manipulated in this way include Binjari virus for flavivirus vaccines[26] and Eilat virus for a CHIKV vaccine[62]. Binjari virus was used to generate a JEV vaccine that was recently successfully used to vaccinate pigs[63]. For these chimeric ISV-based vaccines, the lack of viraemia in vaccine recipients[26] substantially limits the capacity for transmission to mosquitoes.

RNA vaccines. The emergence of mRNA vaccine technologies as an alternative to more traditional protein-based vaccines has led, for example, to early-stage clinical trials of mRNA vaccines for ZIKV (Supplementary Table 1) and CHIKV[64]. For alphaviruses, mRNA vaccines encoding C–E3–E2–6K–E1 can be used[64]. For flaviviruses, mRNA vaccines[65] and chimeric vaccines[66] have so far tended to use prM–E rather than C–prM–E, as capsid needs to be cleaved from prM–E (achieved during infection by the viral NS2B–NS3 protease) before particle assembly can occur. The absence of capsid protein in the vaccine immunogen may reduce the self-assembly of fully authentic virus particles; for example, JEV prM–E generates particles of ~20 nm in diameter as distinct from the normal virus particles of ~50 nm in diameter[67]. In vitro studies also suggest that VLP production may be diminished in the absence of capsid protein[68]. However, prM–E is likely to be widely adopted as the immunogen for flaviviral mRNA vaccines as the absence of capsid has not been associated with significantly diminished protective immune responses[65,68]. The extraordinary rate of progress in the mRNA vaccine field suggests that traditional technologies may ultimately struggle to compete[69]. However, some issues remain, including whether mRNA vaccines will be able to induce the lifelong protective immunity[70] provided, for example, by a single dose of the current live-attenuated YFV vaccine[71].

A new development for RNA vaccines is the use of self-amplifying mRNA vaccines (known as SAM or saRNA vaccines), which encode the non-structural, RNA-dependent RNA replicase genes of TC-83 (the live-attenuated VEEV vaccine) together with the vaccine immunogen to enable host cells in the body to make copies of the mRNA. GEMCOVAC-19 (a licensed COVID-19 vaccine in India; Gennova Biopharmaceuticals), ARCT-154 (a COVID-19 vaccine recently approved in Japan; Arcturus Therapeutics and CSL) and the SEQUIVITY platform (MSD Animal Health) all use this SAM vaccine technology. In the vaccine recipient, the double-stranded RNA replication intermediates that are generated during amplification of vaccine mRNA mimic viral RNA replication and induce innate immune responses involving type I interferon production that may promote long-lasting protective immunity[72,73]. However, the interferon response also inhibits antigen expression, requiring the use of modified nucleotides that both limit interferon production and allow for the first step in RNA replication

Box 1

IXCHIQ, the first approved vaccine for CHIKV

The largest recorded epidemic of chikungunya virus (CHIKV) began in 2004 and spread to more than 100 countries on four continents, causing more than 10 million cases of often debilitating rheumatic disease, with mortality estimates ranging from 0.024% to 0.7%[5]. Several vaccine development programmes ensued (Supplementary Table 1), with the most advanced of these, IXCHIQ (also known as VLA1553), receiving approval by the FDA in 2023 (refs. 111,180) after a pivotal phase III trial involving 3,093 vaccine recipients and 1,035 placebo controls[76]. Following a single vaccination, IXCHIQ demonstrated a 98.9% serum response rate 28 days after vaccination and a 96.3% serum response rate 6 months after vaccination[76]. A phase III efficacy trial in the field was not conducted for IXCHIQ, with efficacy instead being demonstrated by the adoptive transfer of serum from human vaccine recipients to non-human primates that were then challenged with CHIKV[79]. Although this was deemed to provide only low-certainty evidence for protection[111], the Accelerated Approval pathway used in this case allows for the approval of products for serious or life-threatening conditions based on evidence of effectiveness of a product that is reasonably likely to predict clinical benefit. Post-marketing data on adverse reactions and clinical efficacy are required for continued approval[111].

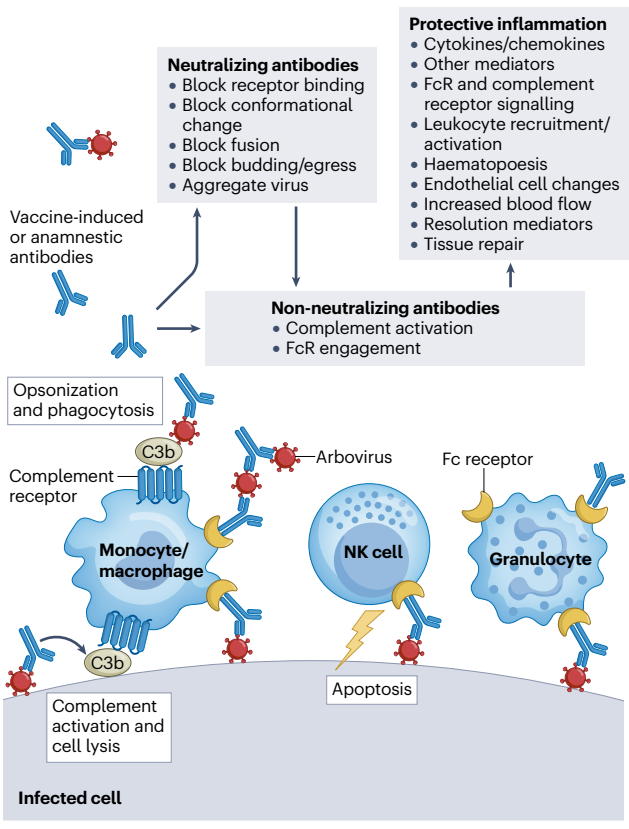
IXCHIQ is a live-attenuated vaccine (propagated in Vero cells) that has a 61-amino-acid deletion in the C-terminal hypervariable domain of non-structural protein 3 of CHIKV. This domain is essential for alphavirus replication but tolerates deletions and insertions[181]. The vaccine is administered as a single intramuscular dose. Levels of viraemia in vaccinated non-human primates are 3–4 logs lower than those seen after infection with the wild-type virus[182], which should also mitigate against transmission to mosquitoes[81].

Box 2

The antiviral mechanisms of antibodies

Virus neutralization is often considered a key determinant of vaccine-induced protection against alphavirus and flavivirus infections and disease[79]. Several mechanisms of antibody-mediated virus neutralization have been elucidated[183]. The 'simplest' of these is the binding of antibody to the viral envelope proteins to prevent their attachment to entry receptors on host cells[184]. MXRA8 (ref. 185) and members of the low-density lipoprotein receptor family[186] have been identified as alphavirus receptors, and DC-SIGN (also known as CD209) and AXL have been identified as candidate receptors for flaviviruses[187]. Other antibody-mediated neutralization mechanisms include virus aggregation[188], cross-linking of envelope proteins[30,189], preventing the structural rearrangements required for fusion with the host cell membrane[30,188,190], or preventing virus budding and egress from host cells[191,192] (see the figure).

These activities of antibodies are generally measured in classical neutralization assays that assess the ability of antisera alone to inhibit virus replication in cell lines in vitro. Antiviral antibodies that have no 'neutralization' activity in vitro but have antiviral activities in vivo are referred to (perhaps incongruously) as non-neutralizing antibodies. Nevertheless, these antibodies can have important protective activities by, for example, activating complement[120,193] or binding Fc receptors (FcRs) on a range of leukocytes (such as natural killer (NK) cells, monocytes/macrophages and granulocytes)[120,194–197]. The FcR-mediated uptake of antibody–virus complexes by antigen-presenting cells and presentation of viral antigen to T cells is also important for the antiviral activity of non-neutralizing antibodies (not shown in the figure), a phenomenon that is illustrated by the boosting of adaptive immune responses by pre-existing antibodies (known as the antibody vaccinal effect)[198]. Neutralizing antibodies can also engage these protective mechanisms in addition to their 'neutralizing' activity; a smaller number of antibodies per virus are potentially needed, for example, to trigger protective FcR-mediated activities than to block virus binding to the host cell receptor[183]. Appropriate inflammatory responses, including those induced by antibody-stimulated, FcR-bearing cells, are often central to an effective protective immune response — potentially involving the production of cytokines, chemokines and other mediators, leukocyte recruitment, vascular changes, and tissue repair responses. However, detailed characterization of protective inflammation, as is increasingly being defined in the context of SARS-CoV-2 infection[199], has received minimal attention for arboviral diseases[200]. Research for arboviral diseases has tended to focus on defining overtly deleterious or pathological inflammatory disease processes[5] rather than on describing the protective inflammatory responses that lead to mild or subclinical outcomes. Pathological and protective responses are likely to be quantitatively and qualitatively different and to have distinct patterns and sequences of immunological events[200,201].



(negative-strand RNA synthesis)[74]. Despite the promise of this technology, SAM vaccines delivering alphaviral C–E3–E2–6K–E1 would likely be deemed too risky as all of the necessary genes would be present to potentially generate a complete, replication-competent, chimeric alphavirus through recombination.

Types of vaccine-induced immune response

Asymptomatic seroconversion rates are high for nearly all flavivirus and alphavirus infections. For example, for JEV, the proportion of individuals who experience asymptomatic infection is usually >95%; for CHIKV, estimates of asymptomatic infection range from 3% to 82%[5]. Asymptomatic or subclinical infections, similar to infections with live-attenuated vaccines[75,76], likely have substantially lower levels of viraemia[77] and lower tissue viral loads (Fig. 1). Thus, for protection from disease, a vaccine does not need to induce sterilizing immunity but only needs to result in antibody levels (early post-vaccination)[78,79] and/or memory responses (likely required later post-vaccination)[72,80] that are sufficient to push the infection towards largely subclinical or mild outcomes. Vaccination-mediated suppression of viraemia would likely also reduce transmission to mosquitoes[81,82].

Alum adjuvants, which are used for many inactivated whole-virus or VLP vaccines, tend to favour T helper 2 (T_H2) cell responses and antibody production over T_H1 cell responses and cell-mediated immunity[55]. By contrast, viral infections and live-attenuated virus vaccines promote

cell-mediated and T_H1 cell responses[83,84]. The paucity of T_H1 cell and $CD8^+$ T cell responses associated with alum-formulated vaccines might be viewed as a disadvantage as these cells often have antiviral activities[85–87]. However, for a vaccine that induces good neutralizing antibody titres and rapid anamnestic antibody responses, it is currently unclear whether the additional induction of antiviral T_H1 cell responses and cell-mediated immunity would result in better performance (for example, with respect to short-term and long-term protection, or reactogenicity).

Post-challenge exacerbation of immunopathology owing to vaccine-induced imbalances in T_H1 cell and T_H2 cell responses has been described for respiratory syncytial virus, SARS-CoV-2 and tuberculosis[88–90]. Thus, a potential theoretical advantage of alum-adjuvanted flavivirus and alphavirus vaccines that induce type 2-biased immune responses may be the avoidance of immunopathological T_H1 cell and/or $CD8^+$ T cell responses. T_H1 cells are thought to be the main drivers of rheumatic disease for arthritogenic alphaviruses such as CHIKV and the Australasian Ross River virus[5]. $CD8^+$ T cells are associated with neuropathology after infection with TBEV[91], ZIKV and WNV[92], and the equine encephalitis viruses[93]. A worst-case scenario for a vaccine that induces T_H1 cell and/or $CD8^+$ T cell responses might be one in which vaccine-induced protective antibody titres wane and, upon challenge, immunopathogenic T_H1 cell and $CD8^+$ T cell responses outpace protective antibody production[94–96]. This issue has not yet emerged in any trials of alphavirus or flavivirus vaccines. Nevertheless, it may need to be considered when mRNA vaccine technology is applied to flavivirus and alphavirus vaccine development[64] given that RNA vaccines have a propensity to induce T_H1 cell-biased and $CD8^+$ T cell-mediated immunity[97,98] and that neutralizing antibody titres may wane quite rapidly after mRNA vaccination[70].

Infection with wild-type virus and with some live-attenuated virus vaccines can provide long-term protective immunity. For example,

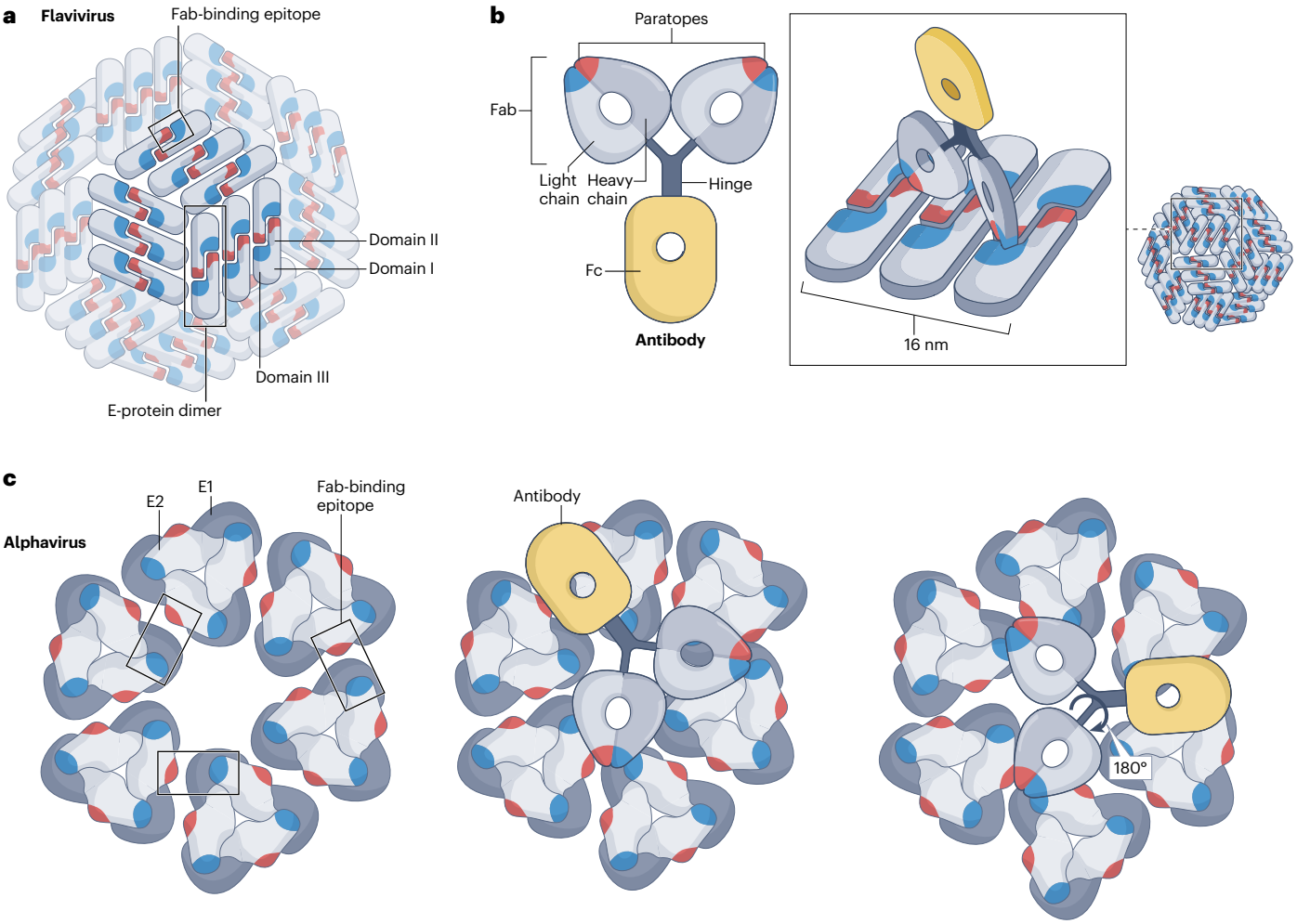


Fig. 2 | Whole virion vaccines and antibody-induced protection. Vaccine immunogens for flaviviruses and alphaviruses are often whole virion structures that provide authentic tertiary and quaternary structures for binding by specific B cell receptors and antibodies. **a**, The pre-fusion arrangement of trimeric envelope (E)-protein homodimers on the flavivirus virion surface is shown, with antibody Fab-binding epitopes indicated in red and blue shading (based on ref. 35). **b**, Binding of an antibody to the flavivirus surface with high avidity (involving both paratopes, each of which has complementary epitope-binding regions indicated in red and blue) can be envisaged (shown at correct relative size). **c**, The same is true for antibody binding to trimeric E1–E2 heterodimers on the alphavirus virion surface (based on ref. 37), with a Fab rotation of 180° also allowing for complementary antibody binding (red on red, blue on blue).

Box 3

Antibody-dependent enhancement of infection

Avoiding antibody-dependent enhancement (ADE) of infection has been a major consideration for dengue virus (DENV) vaccine development[183,202]. For example, a 3-year follow-up for the phase III trial of Dengvaxia showed that vaccinated children >9 years of age had a 1.6-fold increased risk (over placebo) of hospitalization after DENV infection. This could be explained by ADE, assuming that the quadrivalent vaccine induced only subneutralizing antibody titres to one or more of the DENV serotypes in some children. After infection with those serotypes, viral replication and disease would be enhanced via Fc receptor-mediated uptake of antibody complexed with viable (not neutralized) virus. However, the increased risk of hospitalization did not reach significance and there was no evidence of vaccine-induced, post-infection increases in viraemia or pro-inflammatory cytokine levels[203]. No concerns relating to ADE were raised for the phase III trial of Qdenga (ref. 204), for which it might be argued that better quadrivalent neutralizing antibody responses and/or antibody responses to non-structural protein 1 (NS1), induced by the DENV2 backbone, contributed to better protection against all four serotypes of DENV[205]. A single infection with Zika virus was reported to increase the probability of symptomatic and severe DENV2 infection, with ADE being proposed as the mechanism[206]. Some groups thus opted to use Zika virus NS1, rather than prM–E, as the vaccine antigen[87,207,208] because antibodies to NS1 have not been associated with ADE.

ADE can be readily demonstrated in vitro for a large range of viruses, including several flaviviruses, alphaviruses, coronaviruses and influenza viruses[209,210]. However, it is unclear whether ADE represents a major hurdle for future vaccine development for infections other than DENV. For example, pigs vaccinated with a lentivirus vaccine against Japanese encephalitis virus (JEV) prM–E generate sera with strong ADE activity for JEV in vitro but, nevertheless, the vaccine protected pigs against JEV challenge[211]. Similarly, in various settings, ADE of tick-borne encephalitis virus infection can be demonstrated in vitro but enhancement of infection was not observed in vivo[212]. Of note, the possibility that there are different serotypes for SARS-CoV-2 is currently being considered[213] and ADE of SARS-CoV-2 has been widely demonstrated in model systems[214]. However, vaccine-associated ADE for SARS-CoV-2 has not emerged as an important issue despite ~14 billion mRNA vaccine doses administered and ~700 million COVID-19 cases globally[215].

there are no reports of second infections with Ross River virus — a notifiable disease in Australia, with a well-established IgM and IgG testing system[99] — which suggests that a single infection, whether symptomatic or asymptomatic, likely provides lifelong protective immunity[100]. A single vaccination with the live-attenuated YFV vaccine also provides lifelong immunity[71], which is clearly a highly desirable feature, particularly for vaccines to be used in resource-poor settings. One vaccination with the live-attenuated JEV vaccine IMOJEV provided protection for at least 5 years[101]; however, three vaccinations with the live-attenuated DENV vaccine Dengvaxia were required to provide good protection for at least 3 years[102]. Non-replicating immunogens (such as inactivated viruses and VLPs) are often considered less able to provide long-lasting protective immunity[103], although this can be achieved with multiple vaccinations. For instance, GARDASIL 9, a VLP-based human papillomavirus vaccine adjuvanted with alum, provided sustained antibody responses for 10 years after a three-dose regime[104], and clinical benefits from two doses of Shingrix, a recombinant protein herpes zoster vaccine adjuvanted with ASO1B, also seem to last for ~10 years[57]. Our understanding of how long-term humoral immunity is generated is becoming more sophisticated[105,106], with the hope that such knowledge can ultimately be applied to the design of more effective vaccines and adjuvants without increasing adverse event profiles.

Antibody-dependent enhancement of infection is frequently cited as a key issue of concern for vaccine design, although its clinical relevance, particularly for infections other than DENV, may be limited (Box 3). Another vaccine feature that is often discussed is the possibility that serological cross-reactivity might be exploited to allow multiple viruses to be targeted by a single monovalent vaccine (Box 4).

Challenges for phase III human efficacy trials

New and improved vaccines are needed for a range of flaviviral and alphaviral diseases given their substantial social and economic burdens[1,2]. When infection and disease incidence are predictable in specific locations, phase III trials to determine vaccine efficacy are feasible. By contrast, when disease outbreaks tend to be sporadic and unpredictable with respect to season, geographical location and/or patient numbers, such trials become challenging[107]. Deploying multiple trial sites[108] can reduce the risk of having too few cases within the trial period. At the other end of the spectrum, an unprecedented outbreak of the disease during the vaccination process requires methods to distinguish between immune responses owing to vaccination and those owing to infection. For example, if a flavivirus vaccine comprised the structural proteins (C–prM–E), then immune responses to non-structural protein 1 (NS1) could be used to identify infected participants[109]. However, a similar strategy cannot be used for alphaviruses as immune responses to non-structural proteins are generally hard to detect. It may also be difficult to distinguish vaccine-induced adverse events from infection-induced pathology[110,111]. Being infected and also receiving two vaccine doses within a short time frame could, theoretically, augment the severity and incidence of adverse events.

Human challenge models[112], whereby healthy vaccine recipients are challenged with the target pathogen under controlled conditions, have been developed for several pathogens, including malaria[113,114]. Such models have not been widely adopted for testing of flavivirus and alphavirus vaccines owing to safety and ethical concerns related to the absence of licensed and wholly effective antivirals or therapies for flaviviral or alphaviral diseases. Nevertheless, recruitment has recently begun for a controlled human infection model for ZIKV to evaluate escalating doses of two ZIKV strains in 18–40-year-olds in order to establish a challenge model for future ZIKV vaccine testing (ClinicalTrials.gov: NCT05123222). However, it should be noted that ZIKV disease in adults is generally mild (although very rare cases of Guillain–Barré syndrome can occur), with the main burden of disease being associated with congenital Zika syndrome[115].

An alternative and innovative approach to vaccine evaluation is the adoptive transfer of serum from human vaccine recipients into non-human primates that are then challenged with the target

arbovirus[79]. This approach provided some efficacy data for IXCHIQ, the first CHIKV vaccine to be approved (Box 1), and also showed that the recently described cell-to-cell transmission of CHIKV mediated by intercellular extensions[116] may evade neutralizing antibodies but does not prevent antibody-mediated protection in vivo. Adoptive transfer of immune sera from vaccine recipients into mice rather than non-human primates would be considerably cheaper and ethically less contentious but may be less informative. There are considerable differences between the multiple Fc receptors (FcRs) in mice and humans and their binding profiles for the different IgG subclasses[117]. FcR expression patterns by different cell types and associated signalling outcomes also differ between mice and humans[118].

Measuring neutralizing antibody titres in vitro as a correlate of protection is widely used in clinical trials, but titres do not always correlate with protection. For example, in a phase III trial of Qdenga, neutralizing antibody titres against DENV2, DENV3 and DENV4 did not correlate with protection[119]. Such discrepancies likely arise, at least in part, because neutralization assays do not measure the many non-neutralizing, protective activities of antibodies (Box 2). In addition, such assays are usually not standardized, with a wide variety of slightly different protocols used in various laboratories. The requirement for biosafety level 3 containment for many pathogenic flaviviruses and alphaviruses, including JEV, TBEV, YFV, WNV, CHIKV and VEEV, also complicates the use of assays that require live virus (as is the case for most neutralization assays).

The science of 'systems serology' is evolving and promises to provide a more comprehensive characterization of post-vaccination polyclonal antibody responses and their protective capabilities[120]; measurements include antibody isotype and subclass distribution, glycosylation patterns, affinity, repertoire breadth, FcR-binding profile, and complement and phagocytosis activation[121]. 'Systems vaccinology' can provide a comprehensive transcriptomic insight into vaccine responses (usually from peripheral blood) and may also help to predict adverse events such as excessive reactogenicity[122–124]. Advances in these and other technologies, such as biosensing[125], metabolomics[126] and artificial intelligence[127], will hopefully lead to reduced reliance on large phase III efficacy trials by providing licensing authorities with data from vaccine recipients that can more reliably predict the ability of a vaccine to protect against disease.

In summary, although flavivirus and alphavirus vaccines can largely protect recipients from disease, many needed vaccines are not yet available, many people (especially those in resource-poor environments) may not be able to access available vaccines and specific groups of people (such as the young) may be excluded from receiving certain vaccines. Thus, additional measures are needed to control flaviviral and alphaviral diseases; these have primarily focused on controlling viral transmission by mosquitoes, either by reducing mosquito numbers (vector control) or, as discussed below, by generating mosquitoes that are immune to infection.

Prophylactic immunity in invertebrates

Conventional vector control measures (such as insecticides and habitat management) are being challenged by insecticide resistance, mosquito adaptation and the complexities of metropolitan areas[19]. Mosquito transgene technologies are advancing and have provided new opportunities to suppress mosquito populations[128]. For example, Oxitec has developed a transgenic male strain of *Ae. aegypti* (OX5034, Friendly) that expresses a female-specific transcriptional inhibitor of host genes via a tetracycline-off system[129]. Eggs are reared (using tetracycline) and are introduced into a target area. The genetically modified males mate with pre-existing wild female mosquitoes; any female larvae that hatch die before reaching adulthood[130]. In a field trial in dengue-prone neighbourhoods in Brazil, *Ae. aegypti* numbers were suppressed by up to 96%[129]. The approach is species specific and has no off-target impacts (unlike insecticides); however, it requires repeated applications as mosquito populations recover or are re-established through immigration. A more sustainable and potentially cost-effective alternative to vector control is to introduce non-lethal, stable, heritable factors that render mosquitoes resistant to arboviral infections.

The details of insect and mosquito immunity have been reviewed extensively elsewhere[131–133], with key similarities between mammals

Box 4

Vaccines targeting serologically related viruses

Serological cross-reactivity and cross-neutralization between members of the arthritogenic alphaviruses, the equine encephalitic alphaviruses and many pathogenic flaviviruses are well recognized. For example, antisera raised against chikungunya virus recognize other viruses in the same serogroup such as Ross River virus, Mayaro virus and O'nyong nyong virus[59,216]. For the flaviviruses, Zika virus can be recognized by antisera to dengue virus, Usutu virus can be recognized by antisera to West Nile virus (WNV)[217], and Murray Valley encephalitis virus can be recognized by antisera to Japanese encephalitis virus. As case numbers for Ross River virus and Mayaro virus are currently too low for vaccine development to be commercially viable, could a chikungunya virus vaccine, the commercial viability of which is higher, also provide protection against these related alphaviruses? Similarly, could a WNV vaccine protect against Usutu virus[217], or a Japanese encephalitis virus vaccine protect against Murray Valley encephalitis virus?

Mouse studies typically involve high vaccine doses, with dose ranging to find the minimum dose for acceptable levels of protection against the primary target often not undertaken; these high doses often provide encouraging cross-protection data. By contrast, to minimize adverse events, regulators generally favour the minimum dose that provides adequate protection in most human vaccine recipients. To provide adequate cross-protection against serologically related viruses, vaccine doses would likely need to be increased[59]. Although the exact dose increases that would be required remain largely unexplored, the escalating risks of vaccination-associated adverse events would be unlikely to be approved by regulators. Furthermore, pharmaceutical companies may view the small increase in sales resulting from targeting an additional serologically related virus as insufficient to justify the cost of a phase III trial for the additional virus, for which disease prevalence may be low. The tried and tested approach, even for multiple serologically related viruses, is to develop multivalent vaccines[218] such as the trivalent equine encephalitis virus vaccine[11] and the multivalent Core EQ INNOVATOR+V vaccine against six equine infections, including WNV and Venezuelan, Eastern and Western equine encephalitis viruses.

and insects, including conserved innate immune pathways, such as JAK–STAT, Toll, immune deficiency and NF-κB-like (Rel1 and Rel2) pathways, and small interfering RNA (siRNA) pathways that mediate sequence-specific gene silencing through RNA interference (RNAi)[134] (Fig. 3). The key difference is the lack of classical adaptive immunity in insects. Unique features of arbovirus infections in insects include lifelong persistence and, with few exceptions, the absence of observable pathology or reductions in host fitness. Arbovirus infection of mosquitoes is maintained in this chronic, asymptomatic state by the balance between mosquito immunity and arboviral mechanisms of immune evasion[132]. Strategies to promote antiviral immunity in mosquitoes include the use of *Wolbachia* endosymbiotic bacteria, mosquito genetic modifications, ISVs and paratransgenesis, all of which aim to tip the immune balance in favour of virus suppression (Fig. 3).

Wolbachia-infected mosquitoes

Wolbachia is a genus of intracellular bacteria that infects many insects. When introduced into some mosquito species, including *Ae. aegypti*, *Wolbachia* infection disseminates and induces three highly desirable traits: maternal inheritance, whereby infected female mosquitoes pass the bacteria to all of their offspring; cytoplasmic incompatibility, meaning that *Wolbachia*-infected females have a considerable reproductive advantage over uninfected females (as introgression between *Wolbachia*-infected males and uninfected females results in embryonic death); and antiviral immunity, with *Wolbachia* infection markedly reducing virus amplification and transmission of several key pathogens such as DENV, ZIKV, YFV and CHIKV. These three traits allow for the replacement of virus-susceptible, wild-type mosquitoes with virus-refractory populations (Fig. 3a). There is considerable evidence showing the effectiveness of *Wolbachia* for the management of DENV infection, most notably in the Indonesian city of Yogyakarta, where a large randomized cluster trial demonstrated a protective efficacy of 77% across all four DENV serotypes in *Wolbachia*-release areas[135]. A WHO recommendation that recognizes the safety and efficacy of this mosquito population replacement technique is currently in development[136].

All three of the aforementioned traits are, at least partially, driven by the mosquito immune system, although other mechanisms are also involved (such as inhibition of virus replication by competition between viruses and *Wolbachia* for intracellular resources). In established infections, maternal inheritance of *Wolbachia* typically occurs by transmission from mother to offspring through the cytoplasm of ovarian germ cells, which is enabled by the strategies that *Wolbachia* has evolved to evade mosquito immune responses. Such strategies potentially include upregulating or inducing the host cell antioxidant system to counter the production of reactive oxygen species (ROS), inhibiting apoptosis[137,138], and evading cellular defences by compartmentalization of *Wolbachia* in vacuoles associated with the endoplasmic reticulum[139]. In terms of cytoplasmic incompatibility, *Wolbachia* concentrates in the reproductive tissues of its host, where it can manipulate host reproduction and engineer incompatible mating. The *Wolbachia* prophage WO expresses the cytoplasmic incompatibility factor genes *cifA* and *cifB*. These genes induce reproductive incompatibility when *Wolbachia*-infected males mate with *Wolbachia*-naive females. However, the expression of *cifA* in the ovaries of *Wolbachia*-infected females 'rescues' reproductive compatibility[140]. Both genes affect the immune system of mosquitoes by inducing the expression of catalases that attract and degrade ROS, which are key mediators of cytoplasmic incompatibility. In addition, *cifB* encodes proteases (such as Ulp1) that cleave proteins associated with immune-inflammatory pathways such as NF-κB signalling[141]. In terms of antiviral activity, in *Ae. aegypti* (and some other mosquito species), *Wolbachia* reduces viral replication and viral load by various mechanisms, including stimulating ROS production and activating the Toll, immune deficiency and JAK–STAT pathways to increase the expression of downstream immune effectors (such as antiviral peptides) that bind and degrade viruses, including DENV[142]. *Wolbachia* infection also induces expression of a microRNA (aae-miR-2940) that downregulates expression of the DNA methyltransferase *Aa*Dnmt2; *Aa*Dnmt2 promotes DENV replication and its downregulation likely inhibits replication[143].

Transgenic mosquitoes with enhanced immunity

Proof of concept for the stable genetic transformation of mosquitoes was achieved two decades ago with expression of the Toll signalling molecule Rel1 and the antimicrobial peptide defensin[144–146]. However, the first study to demonstrate suppression of arbovirus replication involved mosquitoes engineered to express a long double-stranded RNA encoding inverted-repeat sequences from the prM-encoding region of DENV2 (ref. 147). The double-stranded RNA construct induced the small interfering RNA–RNAi pathway in mosquitoes, which led to silencing of prM expression and effective serotype-specific suppression of DENV2 replication. Transgenic mosquitoes with antiviral effector mechanisms of varying types and efficacies have since been developed[148] (Fig. 3b). These include the transgenic expression of a polycistronic cluster of engineered synthetic small RNAs targeting ZIKV, which is induced after a blood meal, resulting in significantly reduced ZIKV infection and transmission in *Ae. aegypti*[149]. *Ae. aegypti* have also been engineered to express a single-chain variable fragment from a broadly neutralizing monoclonal antibody to DENV, which effectively suppressed the replication and transmission of all four DENV serotypes[150]. Transgenic expression in *Ae. albopictus* of hammerhead ribozymes (RNA motifs that catalyse the site-specific cleavage and ligation of RNA molecules) targeting CHIKV structural protein genes led to inhibition of CHIKV replication[151].

To generate populations of mosquitoes encoding such antiviral effectors, 'gene drive' strategies have been developed[152,153], whereby mating of transgenic mosquitoes with wild-type mosquitoes generates offspring that nearly all encode the antiviral effector (Supplementary Fig. 1). These gene drive strategies are distinct from the self-limiting transgenic technology used, for example, in Oxitec Friendly mosquitoes. Concerns over the release of gene drive-generated mosquitoes include the potential for their unintended geographical spread, the unforeseen spread of the transgene to a new species, and the potential emergence of mosquitoes with new characteristics or capabilities[154]. Such considerations have prompted active discourse of the potential risks (for example, ref. 155) and the development of self-limiting gene drive systems that seek to limit the permanence of transgenes in the environment[156]. So far, no field trials have been carried out of transgenic mosquitoes incorporating gene drive mechanisms. The WHO has released a guidance framework for the testing of transgenic mosquitoes[157], with a range of regulatory and policy considerations, including field testing requirements, post-release monitoring for adverse events and liability issues[158]. It is hoped that this framework will accelerate the assessment of such technologies, which could eventually become game-changing for the control of mosquito-borne diseases.

ISVs to block transmission

ISVs provide another intervention tool that could potentially be used to mobilize insect immunity against pathogenic arboviruses[159–161]. ISVs are

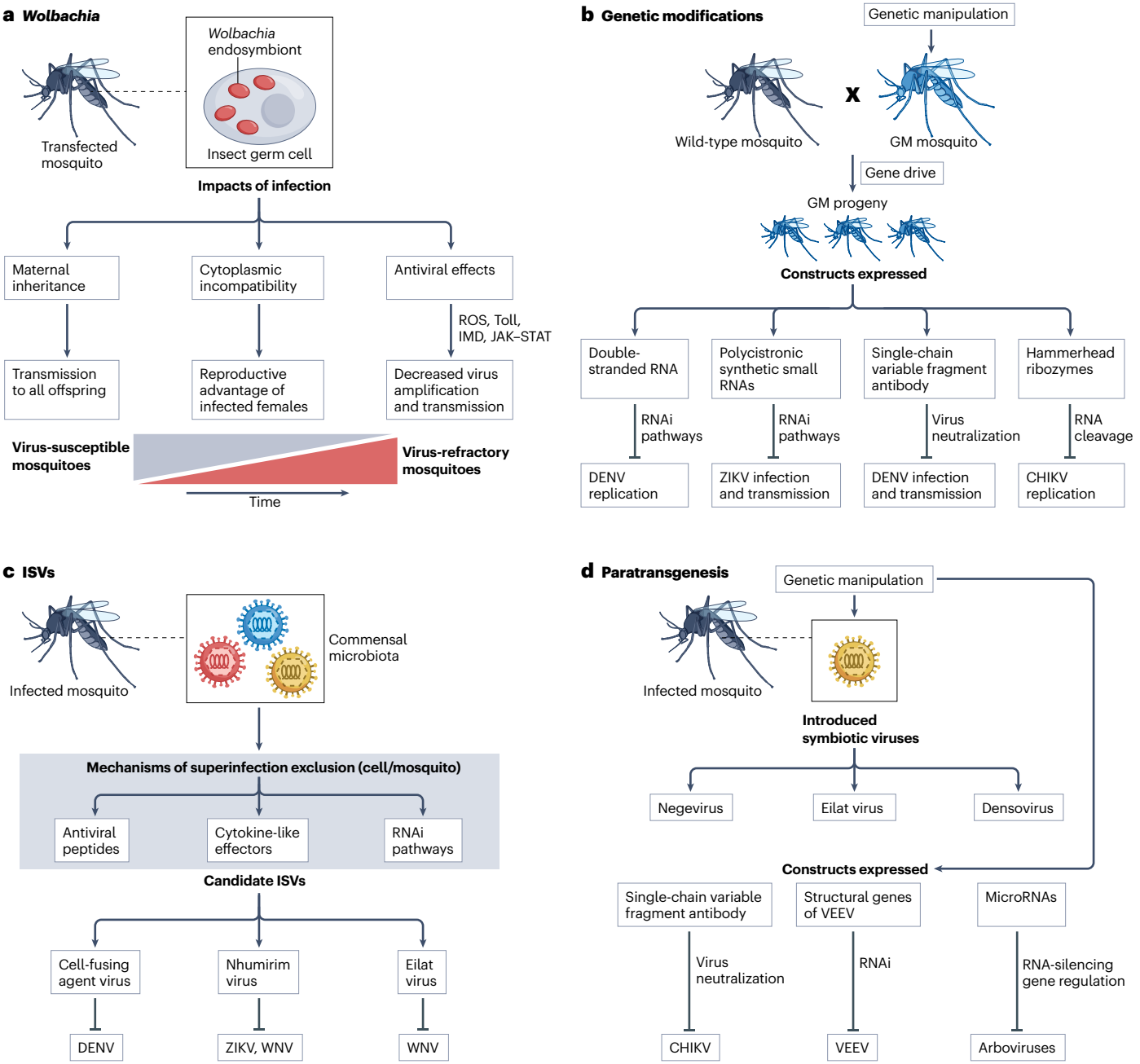


Fig. 3 | Augmenting mosquito immunity to inhibit flavivirus and alphavirus transmission. Details of the mosquito innate immune system have been described previously[131–133] and include the Toll, immune deficiency (IMD), JAK–STAT and RNA interference (RNAi) pathways, and effector molecules (such as reactive oxygen species (ROS)). These elements of the insect immune system can be manipulated by several types of intervention to promote antiviral immunity. **a**, *Wolbachia* endosymbiotic bacteria can be found throughout the mosquito but concentrate in germ cells. Infected female mosquitoes pass the bacteria to all of their offspring (maternal inheritance), and introgression between *Wolbachia*-infected males and uninfected females results in embryonic death (cytoplasmic incompatibility) such that infected females have a reproductive advantage. Infection with *Wolbachia* has effects on the immune system of mosquitoes that decrease flavivirus and alphavirus amplification and transmission. Together, these three features encourage the gradual spread of *Wolbachia* through wild-type populations so that virus-susceptible mosquitoes are replaced by virus-refractory mosquitoes over time. **b**, Mosquitoes can be genetically modified (GM) to express antiviral effector mechanisms of varying types. 'Gene drive' strategies can be used to generate offspring that encode these effector genes. **c**, Insect-specific viruses (ISVs), such as cell-fusing agent virus, Nhumirim virus and Eilat virus, which only replicate in insects, are maintained in mosquitoes via maternal transmission (similar to *Wolbachia*) but there is no reproductive advantage of ISV infection. ISVs can inhibit the replication of pathogenic arboviruses such as dengue virus (DENV), Zika virus (ZIKV) and West Nile virus (WNV) (known as 'superinfection exclusion') by modulating RNAi pathways and by inducing antiviral peptides and cytokine-like effectors. **d**, Symbiotic insect viruses, such as Negevirus, Eilat virus and Densovirus, can also be genetically manipulated (paratransgenesis) to express anti-pathogen effector proteins. These genetically altered viruses are then used to infect mosquitoes. CHIKV, chikungunya virus; VEEV, Venezuelan equine encephalitis virus.

a component of the commensal microbiota of mosquitoes and are maintained in insect populations mainly through maternal transmission. ISVs are being discovered at an increasing rate[26,62], in part owing to the increased application of deep sequencing and metagenomics[162,163]. Certain ISVs can induce whole-organism 'superinfection exclusion', whereby the ISV inhibits infection of the mosquito with a pathogenic arbovirus (Fig. 3c). For example, infection with the insect-specific flavivirus Nhumirim virus reduced infection of *Ae. aegypti* mosquitoes with ZIKV[164] and reduced the number of *Culex quinquefasciatus* mosquitoes capable of transmitting WNV[165]. Inoculation of *Ae. aegypti* with the prototype insect-specific flavivirus cell-fusing agent virus, prior to feeding the mosquitoes DENV-spiked blood, reduced DENV dissemination rates by ~15% and DENV titres by tenfold[166]. In addition, Eilat virus was shown to mediate superinfection exclusion against WNV in *Culex tarsalis* mosquitoes[167]. The mechanisms of superinfection exclusion are poorly understood[168] but may involve RNAi pathways, whereby the ISV and the arbovirus have sequence homology (and infect the same cell), or induction of systemic effectors such as antiviral peptides[169] or the cytokine-like molecule Vago[170].

Challenges facing the use of ISVs for biological control include the need to establish ISVs at high prevalence in mosquito populations. Unlike *Wolbachia*, natural gene drive mechanisms have not been identified for ISVs; although these viruses have been detected at prevalence rates greater than 80% in certain wild mosquito populations[171], the mechanisms responsible remain unclear. Another challenge to address is that certain commensal ISVs may enhance arbovirus transmission as recently demonstrated for two ISVs, Phasi Charoen-like virus and Humaita Tubiacanga virus, which are highly prevalent in *Ae. aegypti* populations worldwide[161]. Infection of mosquitoes with these ISVs blocked the downregulation of histone H4 that is normally seen in DENV-infected mosquitoes, with histone H4 identified as a proviral factor for DENV. Genetic modification approaches can be used to define sequences and mechanisms of ISVs that promote transovarial ISV transmission and prevent arbovirus transmission[169]. This may help in the choice of the best ISV candidates to induce superinfection exclusion and/or lead to the development of genetically modified ISVs with desired properties, although regulatory and oversight issues are likely to be substantial.

Paratransgenesis to express antiviral effectors

Paratransgenesis refers to the genetic manipulation of symbiotic organisms in insects to express anti-pathogen effector proteins[172,173]. Paratransgenesis strategies for mosquitoes were initially developed for the control of malaria parasites[174], with prominent examples being the modification of non-pathogenic *Pantoea* and *Serratia* bacteria to express a range of antiparasitic effector molecules, including a scorpion-derived lytic peptide[175,176]. The modified *Serratia* bacteria inhibited *Plasmodium* infection of mosquito midguts by up to 92% and bacteria were transmitted vertically in mosquitoes for at least two generations. Only a small number of studies have looked at using paratransgenesis strategies to combat arboviruses and these have mainly used ISVs (Fig. 3d). A Negev virus expressing a single-chain variable fragment from an antibody to CHIKV reduced the replication of CHIKV during co-infection in vitro[177]. Chimeric strains of Eilat virus comprising the non-structural genes of Eilat virus with the structural genes of VEEV effectively inhibited VEEV superinfection in vitro through the RNAi pathway[169]. Mosquito densoviruses[162] have been engineered to deliver microRNAs to *Ae. albopictus*[178], a strategy that presumably could be used for arbovirus control.

Key challenges for paratransgenesis approaches include the practical and sustainable delivery of genetically modified ISVs under field conditions as well as, similarly to other approaches, the requirement to develop appropriate regulatory and oversight strategies[172]. Sugar-baited feeding stations provide a potential means of delivery of ISVs but genetically modified organisms that are not vertically or horizontally transmitted within the target mosquito population would need to be continuously delivered, raising major issues relating to effective coverage in the wild and overall sustainability.

Concluding remarks

Arboviral diseases are predicted to increase in prevalence as climate change and urban development accelerate and as the international movement of people and goods increases. Small and/or sporadic market size and/or the difficulties in undertaking phase III efficacy trials for epidemic viruses in resource-poor settings are considerable hurdles for future vaccine development. A key challenge will therefore be to improve our abilities to accurately predict and measure the vaccine-induced immune responses that are sufficient for protection from disease to reduce the reliance on phase III efficacy field trials. mRNA vaccine technology may become the modality of choice for vaccine developers and its application to flavivirus and alphavirus vaccine development is likely to expand. However, the ability of this technology to induce long-lasting protective responses needs to be evaluated for viruses other than SARS-CoV-2 (ref. 64), with new innovations likely to be required[74] to match the durable protection observed for some live-attenuated virus vaccines[71].

Of the novel interventions that seek to suppress or manipulate mosquito populations, two are operationally advanced. Large-scale, government-supported releases of *Wolbachia*-infected *Ae. aegypti* populations are completed, in progress or at the planning stage in 14 countries (Brazil, Colombia, Mexico, Honduras, El Salvador, Indonesia, Laos, Sri Lanka, Vietnam, Australia, Fiji, Kiribati, New Caledonia and Vanuatu)[179]. This technology holds great promise to suppress endemic DENV transmission in many parts of the world and may also help to suppress other viruses transmitted by *Ae. aegypti*. The Oxitec Friendly mosquitoes have been deployed in Sao Paulo, Brazil, with pilot projects in Florida and California in the USA[129]. Currently, their target species is *Ae. aegypti*, which is the ubiquitous vector of a range of arboviruses that are transmitted to humans in urban environments. Both of these interventions have been reviewed by extensive regulatory and consultative processes and have reached a level of public acceptance.

Although stable gene drives for disease-refractory transgenic mosquitoes are now held in several laboratories, concerns around ecological safety and the remediation of releases (that emerge to have unforeseen detrimental outcomes) mean that phase II and III field trials have not been conducted[153]. The potential of ISVs and paratransgenesis to be used for the creation of disease-refractory mosquitoes is well documented, but stably infected mosquito strains with reliable effects on the transmission of human pathogens are not yet available.

The increasing human and economic burden of flaviviral and alphaviral diseases will continue to drive the search for new control strategies. The development, deployment, safety and perception of these initiatives will require more focused collaborations between funders, academia, industry, the media (including social media), educators, and multiple local and international non-government and government agencies.

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Competing interests

A.S. is a chief investigator on a Medical Research Future Fund grant (Australia) and will receive research grant funding from that grant for preclinical evaluation of mRNA vaccines; the principal unvestigator is Southern RNA. A.S. and D.J.R. are chief investigators on an NHMRC development grant application and may receive research grant funding from that grant for preclinical evaluation of chimeric insect-specific virus vaccines; the principal investigator and patent holders are from the University of Queensland. The other authors declare no competing interests.

Additional information

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