

Human immune responses and antibody discovery to West Nile, Powassan, tick-borne encephalitis, and related orthoflaviviruses

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1. Overview

The genus *Orthoflavivirus* (family *Flaviviridae*) comprises a diverse group of arthropod-borne viruses (arboviruses) that are transmitted by mosquitoes and ticks.^{1,2} Members such as West Nile virus (WNV), Zika virus (ZIKV), tick-borne encephalitis virus (TBEV), and Powassan virus (POWV) are of medical relevance because they can cause human disease.³⁻⁶ Their broad geographic location, together with factors such as climate change, global mobility, and changing land-use patterns, has contributed to their circulation in regions where they were not detected before.⁷⁻⁹ Approved vaccines are available only for selected orthoflaviviruses and there are currently no approved antivirals for orthoflaviviral infections,¹⁰ highlighting the importance of developing approaches with wider applicability.

This thesis examines the immune responses that take place during orthoflaviviral infections, with a particular focus on characterizing potent neutralizing antibodies and antibodies that recognize conserved or cross-reactive epitopes shared among different orthoflavivirus family members.^{11,12} Antibodies, which can neutralize multiple orthoflaviviruses are also interesting because they reveal structural elements useful to inform the development of vaccines and therapeutics with broad potential.^{12,13} By mapping these regions and integrating insights from both innate and adaptive immunity, this work aims to inform the design of next-generation countermeasures capable of protecting against both mosquito-borne and tick-borne orthoflaviviruses.^{11,14} In addition, it provides evidence for the presence of orthoflaviviruses in areas where they have not been previously documented, supporting the need for continued monitoring and preparedness. The following sections introduce the biological and immunological background necessary to contextualize these aims.

2. Introduction

2.1 Orthoflaviviruses

2.1.1 General characteristics

The genus *Orthoflavivirus* belongs to the family *Flaviviridae*, which also contains the genera *Pestivirus*, *Pegivirus*, and *Hepacivirus*.^{1,2} Within this family, *Orthoflavivirus* is the largest and most medically important genus, including more than 50 recognized species, many of which can cause severe disease in humans and animals.^{15,16} Orthoflaviviruses are enveloped viruses with a positive-sense, single-stranded RNA (ssRNA) genome.^{16,17} Their distinctive replication cycle across species, epidemiology and immunological properties contribute to the diseases that they cause and to their medical importance, continued emergence, and geographic expansion.^{15,18}

Orthoflavivirus virions are spherical, measuring approximately 40-60 nanometers in diameter, and display an outer lipid bilayer derived from the host cell membrane. Embedded in this bilayer there are two major surface proteins, the envelope glycoprotein (E) and the membrane protein (M), which is processed from the precursor membrane protein (prM). Beneath the envelope lies the capsid protein (C), which packages the viral RNA genome into a nucleocapsid of putative icosahedral symmetry.^{16,19}

The ~11 kilobase (kb) RNA genome is capped at the 5' end, lacks a poly(A) tail, and encodes a single long open reading frame (ORF) flanked by untranslated regions (UTRs).¹⁶ Translation produces a polyprotein that is cleaved by host and viral proteases into three structural proteins (C, prM/M, E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5). The structural proteins participate in forming the virion, whereas the nonstructural proteins are in charge of replication, assembly, and immune evasion.^{16,20,21}

The genus *Orthoflavivirus* is characterized by considerable genetic diversity and evolutionary plasticity. This diversity is driven by the error-prone activity of the viral RNA-dependent RNA polymerase (RdRp; NS5), which lacks proofreading capability. As a result, orthoflaviviruses replicate as dynamic populations of closely related genomic variants, described as viral “quasispecies”.^{22,23} Such heterogeneity enables rapid adaptation to new hosts and ecological niches and promotes the emergence of neutralization-escape mutants, representing a big challenge for vaccine and monoclonal antibody (mAb) development.^{11,24}

The E glycoprotein is the major determinant of tropism and important for immunity. Structurally, it comprises three domains: domain I (EDI) forms a central hinge, domain II (EDII) contains the fusion loop, and domain III (EDIII) resembles an immunoglobulin-like fold involved in receptor binding.¹⁹ Potent neutralizing antibodies often target epitopes in EDIII or quaternary epitopes formed across dimers of E proteins, whereas the conserved fusion loop of EDII is a common target of cross-reactive but weak neutralizing antibodies.^{11,12,25} The prM protein regulates virion maturation by hiding the fusion loop until cleavage by furin in the trans-Golgi network.^{26,27} Incomplete cleavage results in a heterogeneous population of mature, partially mature, and immature particles, which has direct consequences for both viral infectivity and immune recognition, since prM-containing virions can be bound by non-neutralizing antibodies that can mediate antibody-dependent enhancement (ADE).^{25,28,29} This maturation-dependent heterogeneity also affects the earliest steps of infection, because attachment and internalization depend not only on the viral E protein but also on the specific host-cell factors available at the cell surface.²⁹⁻³³

Orthoflaviviruses do not rely on a single universal receptor; instead, they can engage a range of attachment factors and entry receptors whose usage varies by virus, cell type, tissue, and host species.³⁴⁻³⁹ In this context, EDIII is particularly relevant because it contributes to receptor engagement,⁴⁰⁻⁴³ whereas the exposure of EDII fusion-loop epitopes, prM, or antibody-bound surfaces can redirect virions toward alternative uptake routes, including Fcγ receptor-mediated entry during ADE.^{29,44-48} Initial virion concentration at the cell surface can be mediated by glycosaminoglycans such as heparan sulfate, which has been shown to bind to dengue virus (DENV) E protein as well as yellow fever virus (YFV), and Japanese encephalitis virus (JEV) attachment or infection.⁴⁹⁻⁵¹ In WNV and JEV, glycosaminoglycan-binding variants map to determinants in the E protein and show attenuation phenotypes, indicating that glycosaminoglycan

binding may influence tropism and virulence but should be interpreted cautiously as a context-dependent attachment phenotype rather than as evidence for a universal physiological receptor.⁵² C-type lectins provide another well-characterized route of attachment in myeloid, endothelial, and lectin-expressing cells: DC-SIGN (CD209) mediates infection of human dendritic cells by all four DENV serotypes and functions as an attachment factor for JEV through the recognition of high-mannose glycans on E, whereas L-SIGN/DC-SIGNR (CLEC4M) can promote DENV infection in cells expressing this lectin.^{37,53-55} For WNV, DC-SIGNR/L-SIGN promotes attachment and infection more efficiently than DC-SIGN, particularly when WNV is produced in human cells, and the presence of an N-linked glycan on prM or E is sufficient to support DC-SIGNR-mediated infection.⁵⁶ DC-SIGN also enhances infection by glycosylated WNV strains in vitro, and WNV replication in human dendritic cells induces IFN- α and TNF- α production.⁵⁷ In macrophages, the mannose receptor binds the E glycoprotein of all four DENV serotypes and contributes to infection, while CLEC5A acts mainly as a signaling receptor that cooperates with high-avidity C-type lectin interactions to amplify inflammatory responses.^{38,58} Several studies also support an “apoptotic mimicry” route in which exposed virion phosphatidylserine is recognized directly by TIM receptors or indirectly by TAM receptors through bridging ligands such as Gas6 and protein S; however, these factors should be interpreted as context-dependent entry enhancers, since AXL can mediate ZIKV entry in human glial cells but AXL/TAM receptors are dispensable in other cellular or animal models.^{39,59-62} Other proposed orthoflavivirus receptor or receptor-complex components include the high-affinity laminin receptor, GRP78/BiP, and HSP70/HSP90 for DENV in human cells, and $\alpha\beta 3$ integrin for WNV, although integrin involvement has been controversial and cell-type dependent.⁶³⁻⁶⁸ Mechanistically, WNV internalization has been reported to occur through clathrin-mediated endocytosis and Rab5-dependent early endosomal trafficking, whereas another study found that productive WNV entry requires cholesterol-rich membrane microdomains and is independent of $\alpha\beta 3$ integrin, reinforcing that WNV entry route and receptor dependence vary by strain and target-cell context.⁶⁸⁻⁷⁰ In mosquito cells, WNV can exploit a vector-specific receptor system in which the mosquito C-type lectin mosGCTL-1 binds WNV and cooperates with the mosquito CD45-like phosphatase mosPTP-1 to facilitate viral attachment and infection; antibodies against mosGCTL-1 have been shown to reduce DENV attachment and mosquito infection.^{71,72} Importantly, recent studies have expanded the low-density lipoprotein receptor (LDLR)/LRP family as candidate orthoflavivirus entry receptors. LRP1 was shown to bind DENV particles through EDIII of the E protein, and LRP1 depletion reduced infectious virus production, identifying LRP1 as a DENV receptor candidate in mammalian cells.⁷³ For WNV, LDLR was recently identified as an important host factor involved in orthoflaviviral entry and replication in neurons and as a cell-attachment factor that facilitates JEV and WNV uptake.⁷⁴ For TBEV, two independent studies identified LRP8/ApoER2 as an entry receptor that directly binds the viral E protein, promotes attachment and internalization, supports infection by multiple TBEV strains and related tick-borne orthoflaviviruses, and can be targeted by soluble decoy receptors; however, one of these studies specifically reported that LRP8 did not function as an entry

receptor for other tested orthoflaviviruses, including WNV, suggesting specificity within the LDLR/LRP receptor family rather than broad use by multiple orthoflaviviruses.^{75,76} For YFV, other studies reported the LDLR-family, with LRP4 promoting entry through the LDLR type A domains that bind EDIII, LRP1 and VLDLR also acting as entry receptors, and LRP8 functioning as a YFV receptor in human and mosquito cells.^{77,78} Together, these findings suggest that LRP/LDLR-family receptors contribute to orthoflavivirus tropism in a virus-specific manner and further support EDIII as a key receptor-binding surface. In contrast, during ADE, the relevant receptors are Fcγ receptors rather than canonical attachment receptors: subneutralizing IgG–virion complexes can be internalized through FcγR-bearing cells such as monocytes and macrophages, with FcγRIIA/CD32A shown to mediate enhanced DENV infection.^{48,79}

After attachment and receptor-mediated internalization, virions are typically taken up by endocytosis. Acidification of the endosomal compartment triggers the conformational rearrangements of E that drive membrane fusion, allowing release of the viral RNA genome into the cytoplasm.⁸⁰⁻⁸² At this point, the focus shifts from structural proteins, which mediate virion attachment, entry, and fusion, to the nonstructural proteins that replicate the genome, remodel intracellular membranes, and counteract host antiviral responses.

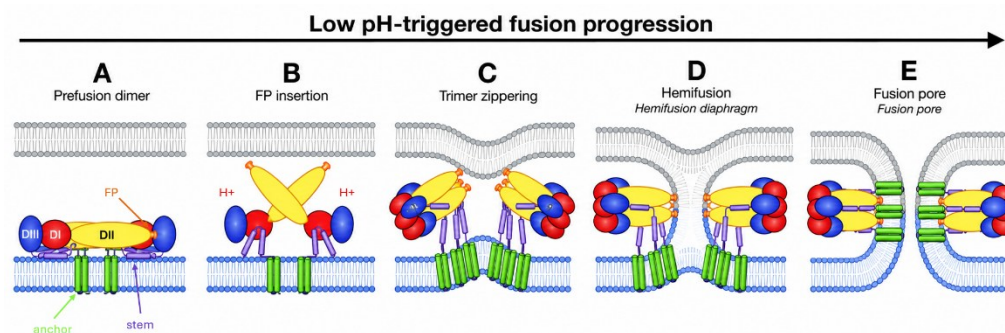


Figure 1. Low pH-triggered orthoflavivirus membrane fusion progression. Schematic model of the fusion process of TBEV. EDI, red; EDII, yellow; EDIII, blue; Fusion peptide (FP), orange; stem, purple; transmembrane anchor, green; viral membrane, blue; target membrane, gray. (A) At neutral pH, E proteins are arranged as prefusion dimers on the viral membrane. (B) Acidification induces dimer dissociation and insertion of the FP into the target membrane. (C) EDIII relocates toward the trimer core during trimer formation, while zippering of the stem pulls the viral and target membranes into close proximity. (D) A hemifusion diaphragm forms, with fusion of the contacting membrane leaflets while the distal leaflets remain separate. (E) Fusion pore opening completes membrane fusion, placing the FP and transmembrane anchor together in the fused membrane. Adapted from Stiasny et al.⁸³

The nonstructural proteins have multiple roles. NS1 exists in intracellular, membrane-associated, and secreted forms. The secreted hexameric NS1 modulates complement activity and contributes to increased vascular permeability and leakage.⁸⁴⁻⁸⁶ As a nonstructural protein that is not displayed on the virion surface, NS1 also represents a distinct antigenic target during infection. NS3 functions as a serine protease (with NS2B as a cofactor), RNA helicase, and NTPase.²⁰ NS5 is the largest protein, carrying both RdRp and methyltransferase activities, critical for RNA synthesis and capping.^{21,87} The

smaller membrane proteins (NS2A, NS4A, NS4B) remodel intracellular membranes to form replication complexes and antagonize interferon signaling.⁸⁸⁻⁹⁰

Orthoflavivirus replication occurs in close association with membrane rearrangements derived from the endoplasmic reticulum (ER), which are induced by viral non-structural proteins.^{16,88,91} Following entry and uncoating, the positive-sense ssRNA genome is translated into a single polypeptide, which is cleaved into structural and non-structural proteins by host and viral proteases. The non-structural proteins assemble into a membrane-associated replication complex that synthesizes a complementary negative-sense RNA intermediate, which then serves as the template for production of multiple progeny positive-sense genomes. Assembly takes place at the ER membrane, where the capsid protein associates with newly synthesized genomic RNA and buds into the ER lumen together with prM and E glycoproteins, to form immature particles. During trafficking through the Golgi, prM is cleaved by the host protease furin, enabling structural rearrangement of the envelope proteins and maturation of infectious virions. Mature particles are released via the secretory pathway.^{26,27}

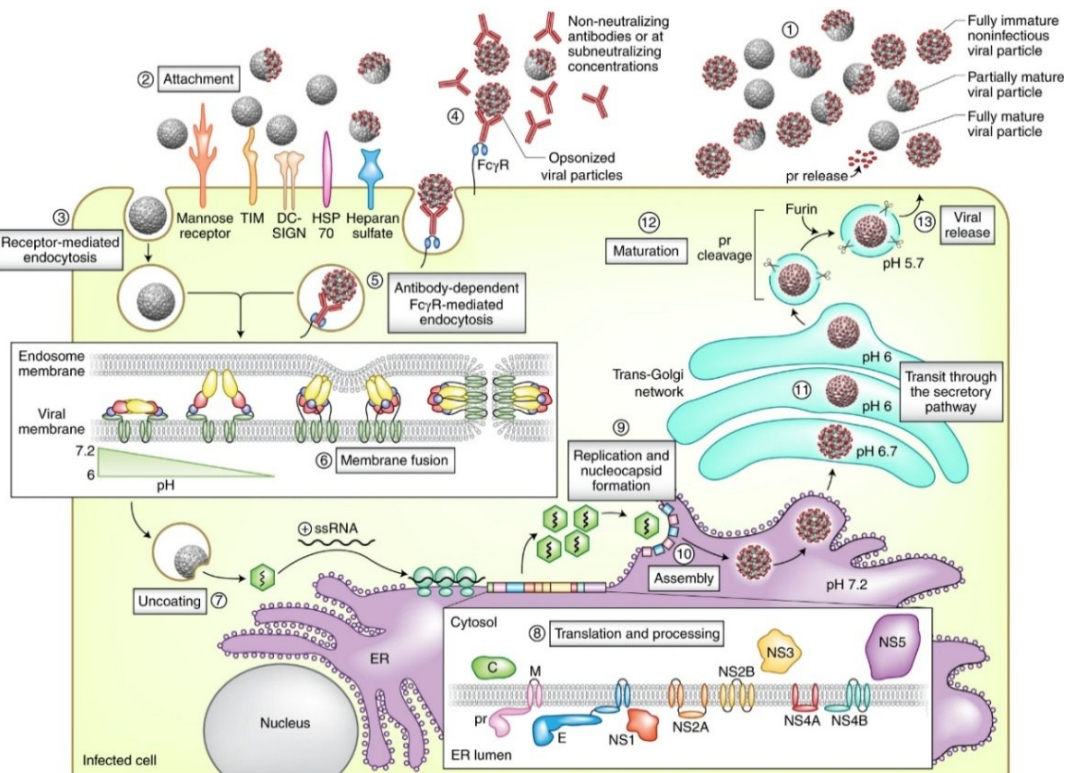


Figure 2. Orthoflavivirus entry, replication, assembly and maturation. Orthoflavivirus infection begins with the attachment of viral particles to the surface of the cells through interactions with attachment factors, followed by engagement of entry receptors that promote endocytic uptake. In the presence of non-neutralizing or sub-neutralizing antibodies, antibody-coated virions may also enter cells through Fcγ receptor-mediated endocytosis. Acidification of the endosome induces conformational rearrangements of the envelope protein, leading to membrane fusion and release of the nucleocapsid into the cytoplasm. The viral RNA genome is then translated and processed, allowing replication to begin. New nucleocapsid molecules assemble at the endoplasmic reticulum, where immature virions bud into the ER lumen and subsequently traffic through the secretory pathway. During passage through the trans-Golgi network, the

acidic environment promotes furin-mediated cleavage of prM, resulting in virion maturation. Mature progeny virions are then released from the cell, with pr dissociating from the viral surface after secretion. Adapted from Slon Campos et al.¹¹ Abbreviations: C, capsid protein; DC-SIGN, dendritic-cell-specific intracellular adhesion molecule-3-grabbing non-integrin; E, envelope protein; ER, endoplasmic reticulum; FcγR, Fc gamma receptor; HSP, heat-shock protein; M, membrane protein; pr, precursor peptide; prM, precursor membrane protein; ssRNA, single-stranded RNA; TIM, T-cell immunoglobulin and mucin-domain protein.

Taxonomically, orthoflaviviruses can be divided into four ecological and phylogenetic groups:^{2,15} the mosquito-borne flaviviruses (MBFVs), the tick-borne flaviviruses (TBFVs), the insect specific and the unknown vector.^{15,92} MBFVs include some of the most globally significant pathogens: dengue virus serotypes 1–4 (DENV1–4), ZIKV, WNV, YFV, JEV, Murray Valley encephalitis virus (MVEV), Saint Louis encephalitis virus (SLEV), and Usutu virus (USUV). TBFVs include TBEV, POWV, Langat virus (LGTV), Louping ill virus (LIV), Omsk hemorrhagic fever virus (OHFV), and Kyasanur Forest disease virus (KFDV). Several insect-specific orthoflaviviruses replicate only in mosquitoes or ticks and based on current understanding do not infect vertebrates.^{93,94}

Within these groups, antigenic variation is considerable. Dengue virus exists as four distinct serotypes, each capable of causing disease and eliciting only partial immune cross-protection against the others.^{95,96} TBEV comprises three major subtypes (European, Siberian, and Far Eastern), with different clinical severities and geographic distribution.^{92,97} WNV also shows genetic and antigenic diversity, with eight reported lineages - lineages 1 and 2 are associated with human disease, differing in geographic distribution, virulence, and immunogenicity.^{98,99} Such diversity underlies the immunological complexity of orthoflavivirus infections: cross-reactive antibodies can be both protective and potentially pathogenic, while vaccines must balance breadth and specificity.^{11,100} Apart from their molecular and antigenic features, orthoflaviviruses are also defined by the ecological interactions that sustain their transmission in nature.

2.1.2 Transmission

The ecology of orthoflaviviruses is defined by their capacity to infect hematophagous arthropod vectors.¹⁰¹ Transmission requires the virus to replicate efficiently in both vertebrate hosts and arthropod vectors, a dual-host cycle that shapes viral evolution.¹⁰¹

MBFVs are primarily transmitted by *Culex* or *Aedes* mosquitoes.^{101,102} *Culex* species maintain bird–mosquito cycles for viruses such as WNV, JEV, MVEV, USUV, and SLEV.^{3,103,104} Birds serve as amplifying hosts, developing high-titer viremia that is enough to infect new mosquitoes, while humans and horses are dead-end hosts due to the low-level or transient viremia.^{3,105} By contrast, *Aedes aegypti* and *Aedes albopictus* maintain primate–mosquito cycles for DENV, YFV, and ZIKV.^{102,106} These viruses are capable of efficient human–mosquito–human transmission, making urban epidemics independent of wildlife reservoirs.^{102,107,108} Vertical transmission to mosquito offspring has been reported for MBFVs including ZIKV, but is generally considered a minor contribution to virus maintenance.^{109,110}

TBFVs follow distinct transmission dynamics.⁶ Ticks such as *Ixodes ricinus* and *Ixodes persulcatus* transmit TBEV in Europe and Asia, while *Ixodes scapularis* and *Ixodes cookei* transmit POWV in North America.¹¹¹⁻¹¹³ Small mammals act as reservoirs and amplifying hosts, whereas large hoofed mammals such as deer and cattle, along with birds, help disperse infected ticks.^{6,111} Unlike mosquitoes, ticks can transmit viruses transstadially (between larval, nymphal, and adult stages).^{114,115} Although transovarial transmission (from female to eggs) has been reported in ticks, it is not considered to be the main route of transmission and maintenance.^{6,116} This capacity allows viruses to persist in tick populations through different seasons, even in the absence of vertebrate amplification, contributing to the long-term stability of TBFV foci.^{111,117}

The geographical distribution of orthoflaviviruses mirrors the ecology of their vectors.^{101,107} DENV is hyperendemic in over 120 countries, with hundreds of millions of infections annually.¹¹⁸ ZIKV, once confined to Africa and Asia, spread explosively through the Americas in 2015–2016.^{108,119,120} WNV, after its introduction into the United States in 1999, rapidly became established throughout North, Central, and South America.^{105,121} In Europe, WNV is now endemic across much of southern and eastern regions and is progressively expanding into central and northern Europe.^{98,122} Warmer temperatures, extended mosquito seasons, and altered precipitation patterns have facilitated outbreaks in countries such as Italy, Greece, Hungary, Germany, and the Netherlands, including areas where WNV activity was rare or absent.¹²³⁻¹²⁵ JEV is endemic across Asia, where it remains the leading cause of viral encephalitis.^{103,126} TBEV circulates widely across Europe and Asia, with rising incidence in northern regions.^{122,124,127} POWV, historically rare, is increasingly reported in North America.¹²⁸⁻¹³⁰ USUV, once restricted to Africa, is now widespread in Europe, often co-circulating with WNV.^{104,131,132}

Climate change and human activity are important drivers for changing these transmission patterns.^{107,124} Rising temperatures and altered precipitation expand mosquito ranges into temperate regions, while milder winters increase tick survival and activity.^{123,124} For example, *Aedes albopictus*, once limited to Southeast Asia, now colonizes Europe and North America, enabling autochthonous transmission of DENV, ZIKV, and chikungunya virus (CHIKV, an alphavirus).^{124,133,134} In northern Europe and Russia, *Ixodes* ticks are expanding into higher latitudes and altitudes, correlating with increased TBEV incidence.^{122,124} For WNV specifically, warmer summers and shorter winters in Europe have prolonged the transmission season and allowed *Culex* vectors to establish in more northern areas, driving recurrent outbreaks in Europe.¹²³⁻¹²⁵

Human-driven ecological changes further amplify transmission risk.¹⁰⁷ Urbanization provides dense human populations and breeding sites for *Aedes* mosquitoes.^{107,135} Deforestation and agricultural expansion increase exposure to wildlife cycles of KFDV and OHFV.^{107,136} Global travel and trade allow rapid dissemination, as evidenced by ZIKV's spread across continents within months during the 2015-2016 epidemic.^{124,137,138} Migratory birds also disperse viruses such as WNV and USUV across long distances, linking outbreak foci along their flyways.^{3,104}

Importantly, virus surveillance is very different between countries, and some regions lack adequate monitoring systems to detect orthoflaviviruses that may already be circulating or to recognize low-level enzootic activity.^{122,139,140} This creates blind spots where orthoflaviviruses such as WNV or TBFVs could persist undetected, delaying outbreak recognition and public health responses.^{122,131} Sustained, coordinated monitoring of vectors, animal hosts, and human populations is therefore critical for early detection, risk assessment, and on timely implementation of control measures.^{122,139}

Thus, orthoflavivirus transmission is dynamic and global, driven by ecological, climatic, and anthropogenic factors.^{101,107,124} Their dual-host cycle and capacity for ecological adaptation explain both their persistence in endemic regions and their potential for rapid emergence in new regions.^{101,107} Among these viruses, several mosquito-borne orthoflaviviruses are of particular interest because of their neuroinvasive potential, global spread, and relevance to public health.^{105,126}

2.1.3 West Nile virus and related orthoflaviviruses

WNV, is a member of the Japanese encephalitis (JE) serocomplex, that is an example of the encephalitic mosquito-borne orthoflaviviruses.^{105,121} First isolated in Uganda in 1937, WNV caused sporadic outbreaks in Africa, the Middle East, and southern Europe for decades. Its unexpected emergence in New York City in 1999 marked a turning point in arbovirology: in few years, WNV had spread across North America, causing tens of thousands of human cases and establishing endemic transmission from Canada to parts of Latin America and the Caribbean.^{105,141,142}

In humans, most WNV infections (~60-80%) are asymptomatic. Around 20% manifest as West Nile fever, characterized by fever, rash, and myalgia, while fewer than 1% progress to neuroinvasive disease, developing meningitis, encephalitis, or acute flaccid paralysis. Mortality rate of 4–14% is present in neuroinvasive disease cases and within these cases, survivors often present long-term neurological sequelae.^{105,143} Risk factors for severe disease include advanced age, CCR5 deficiency, caused by the CCR5Δ32 polymorphism, immunosuppression, and chronic comorbidities.^{105,144-146}

Epidemiologically, WNV is maintained in bird–mosquito cycles, with *Culex* mosquitoes being the principal vectors and birds the main amplifying hosts. Spillover to humans and horses occurs seasonally, during peak mosquito activity. Epidemic amplification is influenced by bird migration, mosquito abundance, and climatic factors.^{3,105} Of note, since several years WNV is detected in mosquitoes during surveillance in Ticino, Switzerland, and in 2025 was reported the first case of autochthonous human WNV infection, also in Ticino.

USUV is another member of the JE serocomplex and is closely related to WNV.^{147,148} Originally isolated in South Africa in 1959, USUV remained largely restricted to Africa until its introduction into Europe in the 1990s, where it now co-circulates with WNV in *Culex* mosquitoes.^{104,149,150} USUV is mostly a bird pathogen, causing significant avian mortality in species such as blackbirds and owls, but sporadic human infections have been

reported, mostly presenting as mild febrile illness or asymptomatic viremia in blood donors.^{147,151} Rare cases of neuroinvasive disease have also been documented, highlighting its pathogenic potential.^{151,152} The strong antigenic similarity between USUV and WNV complicates serological diagnosis, as cross-reactive antibodies hinder species-specific detection.^{150,151}

JEV is the prototypical member of the JE serocomplex and one of the leading causes of viral encephalitis in Asia.^{153,154} First identified in Japan in the 1930s, JEV is now endemic across large parts of East, South, and Southeast Asia, and has recently expanded into northern Australia.¹⁵⁴ It causes an estimated 67,900 clinical cases annually, with a case fatality rate of 20–30% and permanent neurological sequelae in 30–50% of survivors.^{153,155} Transmission occurs mainly through *Culex tritaeniorhynchus* mosquitoes, which maintain enzootic cycles between waterbirds and domestic pigs. Pigs serve as key amplifying hosts, sustaining high-level viremia and facilitating spillover to humans.^{153,154} Unlike WNV and USUV, JEV is a major vaccine-preventable disease: several safe and effective vaccines are licensed and widely used, dramatically reducing incidence where vaccination programs are implemented.^{156,157}

MVEV is endemic to northern Australia and Papua New Guinea and remains an important cause of arboviral encephalitis in Australia.^{158,159} First recognized during outbreaks in the 1910s–1950s, MVEV circulates in bird–mosquito cycles involving *Culex annulirostris*.¹⁵⁸ Human disease is rare but severe, with a case fatality rate of approximately 20% and frequent neurological sequelae among survivors.^{158,159} Large outbreaks tend to follow periods of heavy rainfall and flooding, which expand mosquito breeding habitats and increase contact between vectors and reservoir hosts.^{158,160}

SLEV is distributed throughout the Americas, from Canada to Argentina.^{161,162} Identified in Missouri, United States of America (USA), in 1933, SLEV shares ecological and clinical features with WNV, including *Culex* vector transmission and avian reservoirs.^{161,163} Human infections are typically asymptomatic or mild, but neuroinvasive disease can occur, particularly in the elderly.^{161,163} SLEV outbreaks are sporadic but can be large, with notable epidemics in the USA during the mid-20th century and more recent re-emergence in South America.^{161,162}

These viruses—WNV, USUV, JEV, MVEV, and SLEV—form the encephalitic mosquito-borne orthoflavivirus cluster and are unified in the JE serocomplex.^{105,147,153} They share key ecological features: maintenance in bird–mosquito transmission cycles, seasonal amplification during periods of high mosquito activity, and the capacity to cause neuroinvasive disease in humans. Their overlapping antigenic profiles present diagnostic challenges due to extensive cross-reactivity of orthoflavivirus antibodies that complicates serologic assays,^{150,164} but also offer opportunities for cross-protective immunity that could inform the development of broadly protective vaccines and therapeutic monoclonal antibodies (mAbs).¹⁶⁵

Although these encephalitic mosquito-borne viruses illustrate one major branch of orthoflavivirus diversity, an equally important group is formed by the tick-borne orthoflaviviruses, which differ in ecology, transmission dynamics, and disease manifestations.

2.1.4 Tick-borne encephalitis, Powassan, and related orthoflaviviruses

TBEV is the prototypic TBFV and a major cause of viral encephalitis in Europe and Asia.^{92,166} It comprises three main subtypes: European, Siberian, and Far Eastern. Clinical infection is often biphasic. The first phase resembles influenza, with fever, malaise, and myalgia. After an asymptomatic interval, a second phase may involve the central nervous system (CNS), leading to meningitis, encephalitis, or meningoencephalomyelitis. Case fatality rates vary from 1–2% in the European subtype to 20–30% in the Far Eastern subtype.^{92,166} Survivors frequently suffer long-term sequelae, including cognitive impairment, ataxia, and paralysis.⁹² Vaccines based on inactivated TBEV are licensed in Europe and Russia and provide effective protection.¹⁶⁷⁻¹⁶⁹

POWV is the only known tick-borne orthoflavivirus endemic to North America.^{170,171} Human cases were historically rare but reports have increased in recent decades, particularly in the northeastern United States and Canada.¹⁷⁰ Uniquely, POWV can be transmitted within minutes of tick attachment, in contrast to the hours typically required for transmission of bacterial tick-borne pathogens.^{170,171} Clinically, POWV causes severe neuroinvasive disease with mortality of ~10% and long-term neurological impairment in over half of survivors.^{170,171}

Other TBFVs illustrate ecological and clinical diversity. LGTV is generally nonpathogenic and has been used experimentally in live-attenuated vaccines, but development was limited by residual neurovirulence and safety concerns.⁵ LIV causes encephalitis in sheep and grouse in the British Isles, with occasional zoonotic infections.¹⁷² OHFV in Russia and KFDV in India cause hemorrhagic syndromes rather than encephalitis, with case fatality rates of 3–10%.^{5,173}

TBFVs are sustained by Ixodes ticks, which serve as both vector and reservoirs, with long lifespans and overwintering capacity.^{5,166} This ecological resilience underlies the persistence of focal endemicity and complicates control measures. In contrast to these predominantly tick-borne neurotropic viruses, other orthoflaviviruses such as ZIKV, DENV, and YFV are transmitted mainly by *Aedes* mosquitoes and are associated with a broader spectrum of clinical syndromes, including congenital, hemorrhagic, and epidemic disease.

2.1.5 Zika virus and related orthoflaviviruses

ZIKV, discovered in Uganda in 1947, remained hidden for decades until outbreaks in Micronesia (2007), French Polynesia (2013), and the Americas (2015–2016) revealed its epidemic potential.^{174,175} ZIKV is transmitted primarily by *Aedes aegypti* and *Aedes*

albopictus, but also uniquely by sexual, vertical (mother-to-child), and transfusional routes, distinguishing it from most orthoflaviviruses.^{176,177}

Clinically, most ZIKV infections are asymptomatic or produce mild fever, rash, arthralgia, and conjunctivitis. However, the American epidemic revealed severe complications: including congenital Zika syndrome (CZS)—characterized by microcephaly, brain malformations, and neurodevelopmental disorders—and Guillain-Barré syndrome (GBS) in adults.^{178,179} These outcomes highlighted ZIKV's neurotropism and its capacity to trigger autoimmune pathology.^{178,180}

ZIKV belongs to the Spondweni serocomplex and is antigenically related to DENV and YFV, leading to extensive antibody cross-reactivity that complicates serological diagnosis and immune responses.^{181,182} Cross-reactive antibodies can alter immunity to subsequent infections with related orthoflaviviruses, and in the case of DENV, contribute to ADE of disease severity.^{28,183,184}

DENV is the most widespread mosquito-borne orthoflavivirus and a major global health concern. It exists as four antigenically distinct serotypes (DENV1–4), each capable of causing disease and eliciting only partial and temporary cross-protection against the others.¹¹⁸ Infection can range from a mild, self-limited febrile illness (dengue fever) to severe manifestations such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), which are associated with high morbidity and mortality.^{185,186} Transmission is maintained primarily by *Aedes aegypti* in urban environments, with *Aedes albopictus* serving as a secondary vector in peri-urban and rural settings.^{118,187} Dengue is now endemic in more than 100 countries, with hundreds of millions of infections annually, and its geographic range continues to expand due to climate change, urbanization, and increased human mobility.¹¹⁸ Dengue vaccine development illustrates the difficulty of inducing protection against all four DENV serotypes while avoiding vaccine-associated side effects in seronegative individuals. The first licensed dengue vaccine, CYD-TDV (Dengvaxia), protects individuals with previous DENV exposure but is associated with an increased risk of hospitalization and severe infection in seronegative recipients, which restricted its use to individuals with confirmed prior DENV infection.¹⁸⁸ Current dengue vaccine options include TAK-003/Qdenga, a two-dose live-attenuated tetravalent vaccine that has been licensed in multiple countries and the World Health Organization (WHO)-prequalified, and TV003-derived Butantan-DV, a single-dose live-attenuated tetravalent vaccine licensed in Brazil for individuals aged 12–59 years.¹⁸⁹⁻¹⁹² Nevertheless, dengue vaccination remains constrained by serostatus, infecting serotype, age- and region-specific recommendations, and ongoing pharmacovigilance.

YFV is the prototypical orthoflavivirus and the first for which an effective vaccine was developed. Endemic to tropical regions of Africa and South America, YFV is maintained in both wildlife (monkey–mosquito) and urban (human–mosquito) cycles.^{193,194} Human infection can cause a spectrum of disease, from mild febrile illness to severe hemorrhagic fever with multi-organ failure, carrying case fatality rates of up to 50% in severe cases.^{193,195} Control of YFV relies on vector management and widespread use of the live-

attenuated 17D vaccine (YFV-17D), one of the most successful vaccines ever developed, which provides long-lasting protection after a single dose.^{196,197} Despite this, yellow fever continues to cause periodic outbreaks, particularly in areas where vaccination coverage is insufficient or where urban *Aedes* populations are abundant.^{193,198}

Collectively, ZIKV, DENV, YFV, and related mosquito-borne orthoflaviviruses illustrate the clinical and ecological spectrum of orthoflavivirus infections—from self-limiting febrile illness to neuroinvasive disease, hemorrhagic fever, and congenital syndromes. Their overlapping antigenicity presents significant challenges for diagnostics and vaccine development, while their continued spread underscores the importance of integrated vector control, vaccination programs, and sustained surveillance.^{165,193,199}

2.1.6 Next possible orthoflavivirus threats

In addition to well-established human pathogens such as DENV, WNV, JEV, TBEV, and ZIKV, several lesser-studied orthoflaviviruses have been increasingly recognized as potential future threats due to expanding vector ranges, environmental changes, and increased human–vector contact.^{15,165,200} Among these, Spondweni virus (SPOV), Rocio virus (ROCV), Wesselsbron virus (WSLV), and Ilheus virus (ILHV) have historically circulated in geographically restricted enzootic mosquito–vertebrate transmission cycles but have demonstrated the capacity to infect humans.^{15,200} ROCV, for example, caused a large outbreak of severe meningoencephalitis in Brazil, emerging as a significant human pathogen.^{201,202} ILHV has similarly been associated with febrile illness and occasional neurological disease in Latin America.^{203,204} SPOV, the closest known relative of ZIKV, and WSLV, which infects both livestock and humans, further illustrate the range of orthoflaviviruses capable of crossing species barriers and occupying ecological niches similar to other medically relevant orthoflaviviruses.^{15,175}

These observations reinforce the importance of continued surveillance and research to improve preparedness for potential future emergence. At the same time, preparedness depends not only on detecting emerging orthoflaviviruses, but also on understanding the host immune responses that determine whether infection is controlled, cleared, or progresses to severe disease.

2.2 Immune responses to orthoflaviviruses

The outcome of orthoflavivirus infection is determined by the complex interplay between the virus and the host immune system.^{11,205} The human immune system provides multilayered defense mechanisms that act in a coordinated manner to detect, contain, and eliminate viral pathogens while shaping long-term protection. These defenses are classically divided into innate and adaptive components, which operate sequentially but are highly interconnected.^{11,206}

The innate immune system serves as the first line of defense against orthoflavivirus infection. At the site of inoculation, tissue barriers and resident innate immune cells help limit early viral spread. Upon infection, pattern-recognition receptors (PRRs), including Toll-like receptors (TLRs) and RIG-I–like receptors (RLRs) expressed by dendritic cells

(DCs), macrophages, and epithelial cells, detect viral RNA and trigger antiviral signaling.²⁰⁷⁻²⁰⁹ This recognition induces the production of type I interferons (IFN-I) and other inflammatory cytokines, which stimulate the expression of IFN-stimulated genes (ISGs) that establish an antiviral state that restricts viral replication.²⁰⁹⁻²¹¹ Innate immune cells, including natural killer (NK) cells, further contribute to early viral control by eliminating infected cells and producing antiviral cytokines such as IFN-gamma (IFN- γ).^{210,212,213} In addition to limiting viral spread, these responses promote antigen presentation and the recruitment and activation of antigen presenting cells (APCs) and lymphocytes, thereby initiating and shaping adaptive immune responses.^{206,214} In orthoflavivirus infections, early IFN responses are critical for controlling viral dissemination and preventing neuroinvasion; however, these viruses have evolved multiple mechanisms to evade or antagonize IFN signaling, facilitating viral replication and pathogenesis.²¹⁵⁻²¹⁸

The adaptive immune system provides highly specific and long-lasting protection through coordinated humoral and cellular responses. Virus-specific B cells differentiate into plasma cells that secrete antibodies capable of neutralizing virions, blocking cellular entry, and promoting viral clearance through fragment crystallizable (Fc)-mediated effector mechanisms such as opsonization and complement activation.²¹⁹ In parallel, CD4⁺ T helper cells orchestrate antiviral immunity by providing essential signals for B cell activation, affinity maturation, and memory formation, and by producing cytokines that enhance antiviral defenses.^{11,220} CD8⁺ cytotoxic T lymphocytes (CTLs) recognize viral peptides presented on the major histocompatibility complex (MHC) class I molecules and eliminate infected cells, thereby limiting viral replication and spread.^{221,222} Together, these responses contribute to viral clearance and the establishment of long-lived immunological memory that enables rapid and effective responses upon re-exposure.^{11,206} Neutralizing antibodies are key correlates of protection for several orthoflaviviruses and form the basis of effective vaccines such as the YFV-17D and JEV vaccines,²²³⁻²²⁵ while T cell responses play essential roles in viral clearance, immune regulation, and shaping the magnitude and durability of protective immunity, particularly during primary infection and heterologous secondary exposures.^{11,220,222}

For orthoflaviviruses, effective immune responses are essential not only for controlling the early symptoms during acute infection but also for preventing severe disease manifestations, including neuroinvasive disease, hemorrhagic fever, and congenital infection.^{11,226} At the same time, the close antigenic relationships among viruses within this genus create unique immunological challenges. Preexisting antibodies or T cell responses generated during prior orthoflavivirus exposure can confer cross-protection but may also influence disease outcomes by altering viral clearance, immune activation, or causing immunopathology.^{11,227-229} These complex interactions highlight the importance of both the quality and balance of immune responses in determining protection versus disease.

Understanding the coordinated roles of innate and adaptive immunity is therefore fundamental for the development of effective vaccines and antibody-based interventions. The following sections examine these two arms of the immune system in greater detail, with particular emphasis on antibody-mediated immunity and the structural and functional determinants that govern antibody recognition, cross-reactivity, and neutralization of orthoflaviviruses.^{11,219,223}

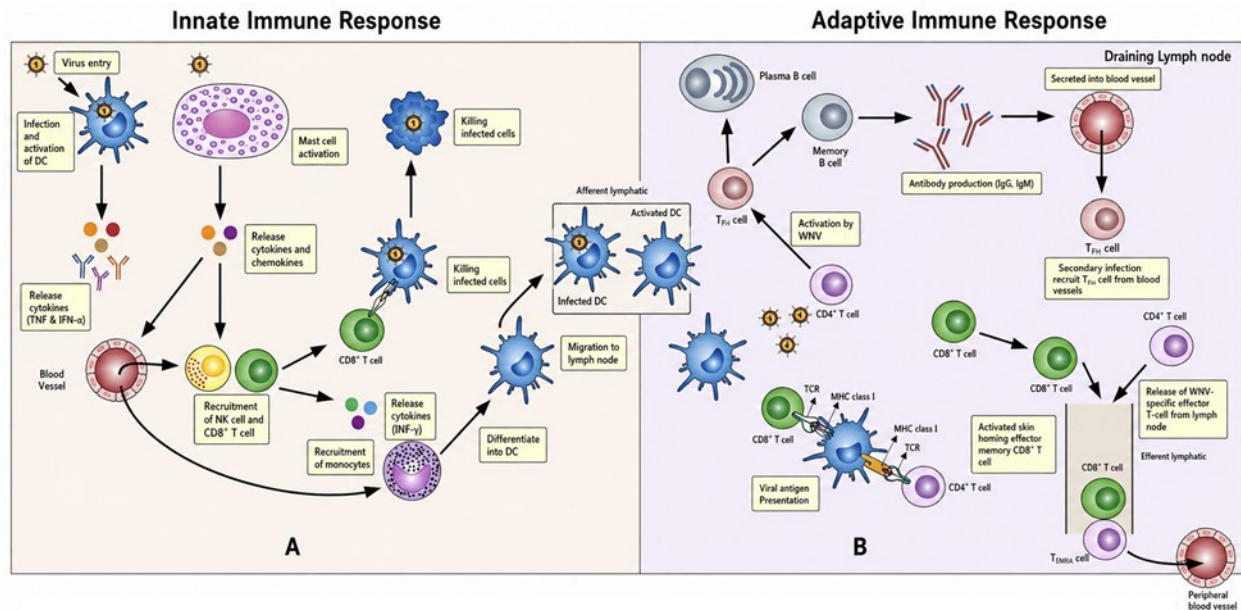


Figure 3. Innate and adaptive immune responses during WNV infection. The immune response to WNV begins when the virus enters the skin during mosquito feeding. At the site of infection, resident dendritic cells and mast cells are activated, initiating the innate immune response. Activated mast cells release cytokines and chemokines that promote the recruitment of natural killer cells, neutrophils and monocytes, which contribute to early viral control. Following skin infection, infected dendritic cells migrate to the draining lymph nodes, where they upregulate co-stimulatory molecules and present viral antigens to CD4⁺ and CD8⁺ T cells. This process activates antigen-specific T-cell responses and links innate and adaptive immunity. Activated T cells then differentiate into effector cells and support the generation of WNV-specific memory B cells and antibody-producing plasma cells within lymph node germinal centers. Adapted from Rahman et al.²³⁰

2.2.1 Innate immunity

The innate immune system provides the body's immediate, nonspecific defense against invading pathogens.¹⁴ Unlike adaptive immunity, which relies on somatically rearranged antigen receptors, innate immunity depends on germline-encoded PRRs and effector mechanisms that respond rapidly within minutes to hours of infection.^{14,231} Physical barriers, soluble mediators such as IFNs, complement, a proteolytic cascade that enhances pathogen clearance, cytokines and chemokines, and sentinel cells—including DCs, macrophages, and NK cells—work together to detect and contain viruses while coordinating immune cell recruitment and shaping the development of adaptive immunity.²³²

Orthoflavivirus infection typically begins at the arthropod bite site, where the virus encounters resident skin defenses.¹⁴ When a mosquito or tick feeds, it injects saliva rich in anticoagulants, vasodilators, and immunomodulatory proteins that facilitate blood feeding and enhance viral establishment. These salivary factors recruit and modulate innate immune cells, including monocytes, macrophages, DCs, and neutrophils, suppress local antiviral responses, and increase vascular permeability, thereby facilitating viral establishment.²³³⁻²³⁵ Initial viral replication occurs in keratinocytes, fibroblasts, tissue-resident macrophages, and dermal DCs, which sense infection and initiate inflammation.¹⁴

From the skin, virus-infected DCs and monocytes migrate through lymphatic vessels to regional lymph nodes, guided in part by chemokine gradients. In these lymph nodes, which are the primary sites of antigen presentation and lymphocyte activation, further replication can occur, viral antigens are presented to lymphocytes, and adaptive immune responses are initiated.^{214,236} Viremia follows, allowing dissemination to secondary organs such as the spleen, liver, and, for neurotropic viruses, the CNS.¹⁴ At each stage of this journey (skin, lymphoid tissue, bloodstream, and target organs) distinct innate mechanisms (e.g., local IFN production, complement activation, NK cell cytotoxicity) act to limit spread and signal the adaptive immune system.^{14,232}

Within infected cells, orthoflavivirus replication generates viral RNA species that act as pathogen-associated molecular patterns (PAMPs). Cytosolic RNA sensors such as RIG-I and MDA5, and endosomal TLRs, TLR3 and TLR7, detect viral RNA and initiate antiviral signaling through adaptor proteins such as MAVS and TRIF.^{14,207} These pathways activate transcription factors IRF3, IRF7, and NF- κ B, inducing the production of IFN-I (IFN- α/β), proinflammatory cytokines such as tumor necrosis factor alpha (TNF- α), interleukin-6 (IL-6), and interleukin-1 β (IL-1 β), and chemokines including CCL2, CCL3, CCL4, CCL5, and CXCL10, which recruit monocytes, NK cells, and lymphocytes to sites of infection.^{14,207} Several of these chemokines signal through the chemokine receptor CCR5, which plays a critical role in leukocyte trafficking and antiviral defense. Notably, in WNV infection, CCR5 deficiency, particularly the CCR5 Δ 32 genotype, has been associated with an increased risk of symptomatic or neuroinvasive disease.^{144,145}

IFN-I are the central antiviral mediators of innate immunity. By binding to the IFN- α/β receptor (IFNAR) on infected and neighboring cells, they activate the JAK-STAT signaling pathway, inducing ISGs that establish an antiviral state.^{210,237-240} Key ISGs include: IFITM proteins, which restrict membrane fusion,²⁴¹⁻²⁴³ OAS-RNase L, which degrades viral RNA, PKR, which inhibits translation,²⁴⁴⁻²⁴⁶ and viperin, which disrupts lipid metabolism and replication complex formation,²⁴⁷⁻²⁴⁹ Type III IFNs (IFN- λ) act similarly, particularly at epithelial and mucosal barriers, reinforcing local protection.^{216,250-252}

Innate immune cells amplify these responses. Dermal DCs and Langerhans cells are among the first infected yet remain major producers of IFN and represent key antigen-presenting cells.^{236,253-255} Monocytes and macrophages infiltrate the bite site, producing cytokines but also occasionally serving as viral replication sites.²⁵⁶ NK cells recognize

stressed or infected cells and mediate cytotoxicity,²⁵⁷⁻²⁵⁹ while neutrophils release inflammatory mediators and may act as transient viral reservoirs.²⁶⁰

Orthoflaviviruses have evolved potent mechanisms to evade and antagonize these IFN pathways. For DENV and ZIKV, NS5-mediated antagonism of human STAT2 is well established, whereas WNV and JEV interfere with IFN signaling through similar mechanisms.²⁶¹⁻²⁶⁵ NS4B of WNV and DENV disrupts RIG-I/MAVS signaling, dampening IFN induction.^{266,267} NS1 secreted by most orthoflaviviruses, inhibits complement activation and modulates vascular permeability.^{85,232} TBEV nonstructural protein NS5, antagonizes IFN signaling by inhibiting the activity of TYK2.²⁶⁸ These evasion mechanisms are critical determinants of virulence and influence species tropism.¹⁴

A recent discovery related to IFNs highlights the importance of these cytokines in human disease. Autoantibodies against IFN-I, that 'neutralize' its ability to signal through IFNAR, have been identified in a significant proportion of patients with severe orthoflavivirus infection. Autoantibodies neutralizing IFN-I were detected in approximately 40% of patients with WNV encephalitis, in ~10% of patients with severe tick-borne encephalitis and in individuals with severe Powassan or Usutu virus disease, which is higher than their prevalence in the healthy population.²⁶⁹⁻²⁷² Such antibodies have also been implicated in adverse reactions to the yellow fever live-attenuated vaccine.²⁷³

It has been proposed that through neutralization of IFN-I activity, these autoantibodies compromise early antiviral defenses and increase susceptibility to severe disease. Their presence not only is likely to explain a substantial fraction of severe cases but also underscores the central role of IFN-I in restricting orthoflavivirus infection.^{269,271-273}

The innate immune response not only limits early viral spread but also provides essential signals for the activation of adaptive immunity. IFNs, cytokines, and antigen presentation by DCs promote the activation and differentiation of virus-specific B and T lymphocytes, enabling the transition from rapid innate defenses to highly specific and durable adaptive protection.^{14,222} The following section therefore turns to adaptive immunity, focusing on how coordinated T- and B-cell responses contribute to viral clearance, immune memory, and long-term protection against orthoflaviviruses and can influence vaccine design.

2.2.2 Adaptive immunity

Following inoculation by mosquitoes or ticks, orthoflaviviruses replicate locally and drain to regional lymph nodes, where adaptive immunity is initiated. DCs capture viral antigens and migrate to lymphoid tissues, presenting peptides on MHC molecules to naïve T cells while delivering co-stimulatory and cytokine signals that drive their differentiation.^{14,222,236} IFN-I, produced within hours of infection, enhance antigen presentation, promote DC maturation, upregulate MHC and co-stimulatory molecule expression, and provide essential signals that support T cell priming, T follicular helper cell differentiation, and B cell activation with germinal center formation.^{14,274} Neutralizing autoantibodies to IFN-I—detected in some patients with severe neuroinvasive disease—could disrupt these pathways, delaying T cell priming and germinal center formation and thereby allowing

higher viremia and greater risk of CNS invasion.^{269,271,272} CD8⁺ T cells recognize viral peptides presented on MHC class I molecules and eliminate infected cells through perforin- and granzyme-mediated cytotoxicity. In WNV infection, robust CD8⁺ T cell responses are strongly associated with viral clearance and survival, whereas impaired responses correlate with severe neuroinvasive disease and mortality in experimental models.^{221,275} Similarly, CD8⁺ T cells contribute to the control of ZIKV infection in both peripheral tissues and the CNS and play protective roles in JEV and TBEV infections by limiting viral replication and dissemination.^{222,276} CD4⁺ T cells recognize antigens on MHC II and differentiate into helper subsets; among them, T follicular helper (Tfh) cells provide signals required for germinal center formation, immunoglobulin class switching, and affinity maturation.^{222,277} Dysregulated T cell activity, however, may contribute to neuropathology in encephalitic orthoflavivirus infections.²⁷⁸ Following viral clearance, a subset of antigen-specific CD4⁺ and CD8⁺ T cells differentiate into long-lived memory T cells, including central, effector, and tissue-resident memory populations. These cells persist for years and provide rapid recall responses upon re-exposure, contributing to accelerated viral control and enhanced protection against reinfection.²²²

Within the lymph node, intact virions also reach B cell follicles, where subcapsular sinus macrophages and follicular DCs transfer antigen directly to naïve B cells, seeding early extrafollicular antibody responses that help limit viremia before the germinal center reaction is fully established.²⁷⁹ B cells with antibodies generated through V(D)J recombination in the bone marrow encounter antigen in the lymph node and, with Tfh help, undergo clonal expansion, somatic hypermutation, and class switching to produce high-affinity, isotype-switched antibodies. Upon differentiation to plasma cells they secrete antibodies, including neutralizing antibodies that provide immediate protection, while their differentiation into memory B cells provides a pool of cells that persists long term and that can rapidly differentiate into antibody-secreting cells upon re-exposure, ensuring durable humoral immunity.^{222,277} In orthoflaviviruses infection, neutralizing antibodies typically target the virus E protein, with domain III and quaternary epitopes spanning E dimers serving as dominant site for neutralization.^{13,25} Vaccines such as those for yellow fever, Japanese encephalitis, and tick-borne encephalitis induce such antibodies, correlating with durable protection.²²² By contrast, antibodies directed to the conserved fusion loop are broadly cross-reactive but poorly neutralizing and can mediate ADE. Dengue provides the clearest example, where pre-existing cross-reactive antibodies from a prior infection can enhance secondary infection with a different serotype and increase disease severity.^{28,184,280} ZIKV infection highlights a different risk: although antibody responses protect against reinfection, some patients develop autoantibodies that cross-react with neural antigens and are implicated in GBS, likely through molecular mimicry between viral epitopes and host gangliosides.^{281,282}

A further layer of complexity arises from immune imprinting during sequential orthoflavivirus exposure. Because many orthoflaviviruses share structurally related epitopes, prior infection or vaccination can shape the specificity, breadth, and functional properties of antibody responses elicited by subsequent exposures. Recent work has

shown that prior TBEV vaccination can skew the response to YFV-17D vaccination toward highly cross-reactive antibodies with limited neutralizing capacity and potential to enhance dengue virus infection *in vitro*, illustrating how pre-existing immunity may redirect humoral responses toward immunodominant but suboptimal epitopes with regard to neutralization.²⁸³ By contrast, sequential exposure can also promote beneficial breadth: antibodies from dengue patients with prior exposure to JEV were shown to exhibit broad and potent neutralizing activity against ZIKV, indicating that immune imprinting can, under some circumstances, favor the generation of cross-protective antibody responses.²⁸⁴ These observations are highly relevant to orthoflavivirus vaccine design, as they indicate that prior orthoflavivirus immune history can influence both protection and the risk of immunopathology.

Thus, B cell responses illustrate the paradox of flavivirus immunity—indispensable for protection, yet occasionally drivers of immunopathology.^{25,184} Because cellular immunity is equally important in limiting viral replication and shaping the quality of humoral responses, the next section examines the contribution of T cells in greater detail.

2.2.3 T cells

CD8⁺ CTLs are indispensable for clearing orthoflavivirus infection. After priming, they migrate to infected tissues, including the CNS, where they recognize viral peptides on MHC I and eliminate infected cells through perforin- and granzyme-mediated cytotoxicity while secreting IFN- γ to suppress replication.^{221,275} Their importance is well demonstrated in WNV models, in which depletion of CD8⁺ T cells or loss of perforin impairs viral clearance and worsens outcome.^{221,275} Comparable protective roles have been described for T cells in other orthoflavivirus infections, but the strength of evidence varies by virus and experimental model.^{222,276} CD4⁺ T cells, though not directly cytotoxic, are equally important because they provide help for B cells and sustain CD8⁺ T cell responses. Depletion of CD4⁺ T cells reduces survival in WNV infection and impairs the development of neutralizing antibodies in ZIKV.^{277,285} Protective outcomes correlate with polyfunctional CD8⁺ T cells producing IFN- γ , TNF, and IL-2, whereas exhausted PD-1^{high} phenotypes are associated with severe neuroinvasive disease.²²² Cross-reactive T cells recognizing conserved epitopes can provide partial heterologous protection, but in some contexts, such as secondary dengue infection, they may drive immunopathology through skewed cytokine production.²²⁹

Recent human data further underscore the importance of T cells as independent correlates of protection in orthoflavivirus infection. In a controlled human challenge study, vaccine-induced T cell responses were shown to control acute orthoflavivirus infection even in the absence of detectable neutralizing antibodies, providing direct evidence that cellular immunity can mediate protection when humoral neutralization is limited.²⁷⁶ These findings refine the traditional view that neutralizing antibodies are the sole correlate of protection and support the inclusion of T cell-directed immune readouts in orthoflavivirus vaccine evaluation.^{222,276}

Alongside these cellular responses, humoral immunity plays a central role in protection against orthoflaviviruses, particularly through the generation of potent neutralizing, and cross-reactive antibodies.^{13,25,184} The following section therefore focuses on B cells and the diverse protective and pathogenic roles of the antibodies produced.

2.2.4 B cells

Neutralizing antibody responses are major correlates of protection for several orthoflaviviruses and licensed orthoflavivirus vaccines. Naïve B cells encountering intact virions or complement-opsonized particles in the lymph node follicle receive Tfh cell help and enter germinal centers, where they undergo clonal expansion, somatic hypermutation, and class switching to generate long-lived plasma cells and memory B cells.^{223,277,279,286} Early extrafollicular plasmablast responses release IgM and low-affinity IgG that blunt viremia before germinal center–derived antibodies emerge.^{286,287} In yellow fever vaccinees, neutralizing antibodies can persist for decades, whereas for TBEV vaccination durable protection is typically maintained by booster immunization.^{274,288-290} Memory B cells ensure rapid recall after reinfection or booster vaccination.^{286,291}

Although the lateral ridge of EDIII is a classical and well-characterized target for potent neutralizing antibodies and has therefore been widely explored as a key antigenic region, anti-EDIII antibody titers do not always correlate with neutralization potency. This observation suggests that, while EDIII plays a critical role in protective immunity, additional epitopes across the E protein also contribute to neutralization and viral inhibition.^{13,25,292} Several highly protective antibodies recognize regions outside EDIII, including the EDI–EDII hinge, quaternary epitopes spanning E dimers, the domain I–II hinge region, stem-proximal regions, and the prM/E interface exposed on immature or partially mature particles.^{13,293-297} Antibodies targeting the E dimer epitope (EDE) can neutralize multiple dengue serotypes and even extend cross-reactivity to ZIKV by stabilizing the E dimer in its pre-fusion conformation.^{295,298} In contrast, antibodies directed against the fusion loop in EDII are highly cross-reactive and often relatively weakly neutralizing, and when present at sub-neutralizing concentrations they can promote ADE.^{12,29,292} Other antibodies target the EDI–EDII hinge, where they inhibit the large conformational rearrangements required for membrane fusion, while antibodies against the E stem region can interfere with late entry steps and/or contribute through Fc-dependent effector functions.^{13,296}

Mapping these diverse epitopes has revealed structural “coldspots” for protective immunity across the E protein and informs the design of immunogens that focus antibody responses on quaternary and hinge epitopes while avoiding fusion-loop immunodominance.^{13,283,295} Importantly, this broader epitope landscape does not diminish the relevance of EDIII; rather, it places EDIII within a wider framework of antibody recognition, where it remains a key target for strongly neutralizing antibodies while additional epitopes contribute to the overall protective and cross-neutralizing antibody repertoire.^{25,291,292}

These general principles are illustrated by several mAbs that target distinct epitopes and mechanisms of protection across orthoflaviviruses. The following mAbs illustrate the breadth of protective targets:

E16 (WNV). E16 recognizes the EDIII lateral ridge rather than a quaternary epitope bridging adjacent E molecules and blocks the low-pH-triggered conformational changes required for membrane fusion after viral internalization in endosomes. In mice it protects when administered therapeutically after infection, including after CNS invasion.²⁹⁹⁻³⁰¹

WNV-86 (WNV). WNV-86 binds a potent neutralizing epitope in EDII, it preferentially recognizes mature virions and exhibits extraordinary neutralization potency in vitro and therapeutic efficacy in vivo, illustrating that protective antibodies need not be restricted to EDIII.²⁹⁶

Z004 (ZIKV). Z004 targets a conformational epitope centered on EDIII lateral ridge residues and can protect mice and non-human primates (together with Z021); Fc-modified Z004/Z021 derivatives were specifically developed to reduce Fcγ receptor engagement and ADE risk while preserving antiviral efficacy.^{302,303}

T025 (TBEV). T025 is a potent TBEV-neutralizing antibody that binds the EDIII lateral ridge close to the EDI–EDIII hinge region, shows strong activity across all three TBEV subtypes, and has therapeutic potential in vivo.²⁹¹

P002 and P003 (POWV). These human antibodies, isolated from a donor in Mexico, neutralize POWV lineage I while recognizing an EDIII epitope shared with a broad range of tick- and mosquito-borne flaviviruses. Their discovery in an individual living in Mexico suggests either unrecognized exposure to POWV-like orthoflaviviruses in Central America or unusually broad cross-reactive immunity and underscores the need for expanded serologic and vector surveillance.³⁰⁴

F25.S02 (DENV/ZIKV). A recently characterized broadly neutralizing human antibody neutralizes all four DENV serotypes as well as ZIKV with high potency by binding a conserved epitope at the E-protein dimer interface.³⁰⁵

4G2 (pan-flavivirus). The well-known 4G2 antibody binds the conserved fusion loop of domain II. It is broadly reactive and widely used as a laboratory detection reagent, but it is generally weakly neutralizing against mature virions and can mediate ADE in Fcγ receptor-bearing cells.^{25,29,306}

These examples illustrate that neutralization is not confined to a single structural region and that multiple independent solutions, virus-specific EDIII antibodies, cross-reactive quaternary antibodies, hinge-binding antibodies, and stem antibodies, can each provide robust protection. Virus maturation and dynamic fluctuation of a virus particle's structure over time, where viral proteins (e.g. E protein) transiently shift, open, or expose otherwise hidden regions without the virus disassembling ("virus breathing") influence the accessibility of these epitopes, dictating the stoichiometry of neutralization and the risk of ADE.^{297,307,308}

Antibody escape can occur under the selective pressure of a single mAb. In TBEV infection, distinct combinations of mutations in the envelope protein emerged in the presence of individual EDIII-targeting antibodies, conferring resistance. In contrast, the combination of two antibodies binding non-overlapping epitopes prevented viral escape and enhanced neutralization, highlighting how structural characterization and epitope mapping can inform the rational design of antibody combinations that increase the genetic barrier to escape.³⁰⁹

Protective antibody responses are not restricted to E. NS1, although absent from the virion surface, is expressed on infected cells and secreted extracellularly, where it contributes to vascular dysfunction and immune evasion. This makes NS1 a distinct antibody target that can mediate protection without promoting ADE. Importantly, at least two complementary studies demonstrated that broadly protective anti-NS1 mAbs can neutralize the pathogenic effects of secreted NS1 across multiple orthoflaviviruses and defined the structural basis by which such antibodies block NS1-triggered endothelial dysfunction.^{310,311} These findings support NS1 as an important target for antibody-based therapies and vaccine strategies designed to reduce disease while avoiding ADE associated with virion-directed antibodies.

In addition to direct neutralization and Fc-mediated effector functions, some antibodies can protect through complement-dependent virolysis. Recent work has shown that anti-DENV antibodies that cross-react with ZIKV and recognize virion-associated epitopes can trigger complement-mediated lysis of viral particles, and that this activity was associated with reduced risk of severe dengue manifestations, including thrombocytopenia and plasma leakage.³¹² These findings broaden the functional framework of protective antibody responses in orthoflavivirus infection and indicate that antibody quality is determined not only by neutralizing potency, but also by the capacity to engage additional antiviral effector mechanisms.

Beyond their protective potential, B cell responses can also drive pathology. ADE of disease is best and for now solely demonstrated for DENV at the epidemiologic level.^{28,313-315} The proposed mechanism is through the existence of sub-neutralizing antibodies from a previous heterotypic infection that facilitate Fcγ receptor–mediated virus uptake into monocytes and macrophages, enhancing viral replication in the macrophage and with its activation leading to strong production of inflammatory cytokines that drive pathology.^{184,280} Although ADE has not been demonstrated for WNV, ZIKV, or TBEV in the population, cross-reactive antibodies targeting conserved epitopes such as the E-protein fusion loop or prM can mediate robust enhancement of orthoflavivirus infection in vitro, raising concern for vaccines or therapeutic antibodies that elicit such responses.^{28,29,316,317} Classic examples are the broadly cross-reactive fusion-loop antibody 4G2, which efficiently promotes Fcγ receptor–dependent ADE of multiple orthoflaviviruses, as well as the E53 mAb.^{29,318} Fc engineering provides a practical countermeasure: therapeutic antibodies have been modified to abolish Fcγ-receptor engagement while preserving neutralization. For example, the WNV antibody E16 was

humanized for therapeutic development and engineered with LALA mutations at the Fc to decrease Fcγ-receptor binding and ADE while retaining protective activity, and engineered Zika antibodies such as Z004-GRLR similarly disrupt Fcγ-receptor interactions to prevent enhancement in vitro.^{300,303,319}

Autoantibody formation adds further complexity. ZIKV infection has been associated with GBS, and post-infectious antibody-mediated mechanisms, including possible cross-reactivity with neural antigens, have been proposed, although the precise pathogenic contribution of such antibodies remains incompletely established.^{281,282} In addition, a high prevalence of patients with severe WNV or TBEV infection bear autoantibodies against IFN-I, which likely predisposes them to impaired antiviral immunity explaining their susceptibility to severe disease.^{269,271,272} Thus, the compound effect of both the antiviral antibodies, but also of the autoantibodies against non-viral targets contribute to protection vs immune-mediated pathology during orthoflavivirus infection.

Comprehensive mapping of the epitopes targeted by neutralizing antibodies provides critical guidance for vaccine and therapeutics design. By defining conserved structural “coldspots”, epitope mapping enables rational immunogen engineering to focus immune responses on protective epitopes while avoiding enhancing epitopes such as the fusion loop or prM.^{13,283,295,320} It also helps surveillance for antigenic variation, informing the selection of vaccine strains and therapeutic antibody cocktails capable of remaining effective against emerging variants.^{309,321} Together, these insights reveal the central paradox of orthoflavivirus B cell immunity: antibodies are indispensable for protection and long-term memory, yet their breadth and cross-reactivity can also fuel disease when responses are misdirected. This duality has major implications for intervention design, making it essential to translate immunological and structural insights into safer vaccines, therapeutic antibodies, and integrated prevention strategies.

2.3 Countermeasures against orthoflaviviruses

Vaccination remains the most effective strategy to control orthoflaviviruses. The live-attenuated YFV-17D vaccine sets the benchmark for durability and breadth, eliciting polyfunctional T cells and quaternary-epitope antibodies that provide decades of protection.^{196,197,286} Recent works have also clarified part of the molecular basis of 17D attenuation. Mutations on the E protein and two nonsynonymous substitutions in NS2B that distinguish the vaccine strain from its parental Asibi virus, I37L and I109L, were shown to act cooperatively to mediate attenuation in vivo, with both mutations required to fully prevent key features of YF disease. These findings identify E and NS2B as important determinants of YFV-17D attenuation and provide mechanistic insight into the safety of this live-attenuated vaccine platform.^{322,323} Live-attenuated JEV SA14-14-2 and inactivated TBEV vaccines achieve similarly high efficacy of protection in endemic regions.^{153,166,167} DENV vaccination illustrates the challenge of ADE: the chimeric live-attenuated Dengvaxia vaccine protects seropositive individuals but increases severe disease risk in seronegative individuals and it is now discontinued.¹⁹⁹ Newer dengue vaccines have shifted the field from candidate-stage development toward licensed and

regionally available products. TAK-003, marketed as Qdenga, is a two-dose live-attenuated tetravalent dengue vaccine that has been licensed in multiple countries and WHO-prequalified; WHO recommends TAK-003 for children aged 6–16 years in settings with high dengue burden and high transmission intensity.³²⁴ Long-term follow-up has strengthened the evidence base for TAK-003. At 4.5 years after vaccination, TAK-003 showed durable efficacy and an acceptable safety profile, with protection observed against all four DENV serotypes in previously exposed individuals and against DENV-1 and DENV-2 in dengue-naïve individuals.¹⁸⁹ These data suggest that TAK-003 may provide sustained benefit, although its performance remains influenced by baseline serostatus and infecting serotype.¹⁸⁹ TV003-derived Butantan-DV represents a second major live-attenuated tetravalent dengue vaccine approach. In phase 2 testing, Butantan-DV and TV003 were safe and induced robust, balanced neutralizing antibody responses against all four DENV serotypes.¹⁹⁰ Subsequent phase 3 studies showed that a single dose of Butantan-DV was generally well tolerated and efficacious against symptomatic virologically confirmed dengue,^{191,192} and five-year follow-up reported sustained efficacy against symptomatic dengue caused by DENV-1 and DENV-2 with no long-term safety concerns identified in the trial population.³²⁵ Butantan-DV was licensed in Brazil for individuals aged 12–59 years, supporting its inclusion as a currently licensed TV003-derived dengue vaccine, although its deployment remains subject to national recommendations and pharmacovigilance decisions.^{192,325-327}

No vaccines are currently licensed for WNV, ZIKV, and POWV, but several next-generation candidates are in development, including using mRNA-based platforms. For ZIKV, mRNA vaccines encoding prM-E antigens have advanced to phase 1 clinical testing; in particular, mRNA-1893 showed an acceptable safety profile and induced robust neutralizing antibody responses that persisted for at least one year after vaccination.³²⁸ For DENV, mRNA-LNP vaccines remain at the preclinical stage, but available studies show that modified mRNA constructs can induce neutralizing antibodies, cellular immune responses, and protection in mouse models, supporting the further development of tetravalent mRNA approaches.³²⁹

Recent studies also illustrate how structural epitope knowledge can be translated directly into safer vaccine design. A 2025 epitope-focused vaccine strategy introduced stabilizing mutations into the orthoflavivirus envelope glycoprotein to reduce exposure of poorly protective, ADE-prone epitopes while preserving key neutralizing determinants. In experimental models, this approach improved the induction of protective antibody responses, including antibodies targeting the EDE, while reducing the risk of ADE.³³⁰ These findings provide an important proof of concept for rational orthoflavivirus vaccine design guided by high-resolution mapping of the epitopes recognized by neutralizing antibodies.

Where vaccination is unavailable, insufficient, or for use in populations at high risk of severe disease, mAbs offer a complementary strategy for both prophylaxis and therapy.

MAbs offer a valuable complement to vaccination because they can provide immediate protection, can be used prophylactically or therapeutically after exposure, and can be rationally engineered to improve potency, breadth, half-life, and safety. Across orthoflaviviruses, potent antibodies directed against E have shown that passive immunization can prevent infection, reduce viremia, and, in some models, retain efficacy even after disease has progressed, including in neuroinvasive settings.^{291,299,303} Recent structural and translational work further shows that antibody performance can be optimized through Fc modification to reduce Fcγ-receptor engagement and the risk of ADE, and through selection of conserved quaternary epitopes that support broader neutralization across related viruses.^{300,303,319,321} Together, these advances position mAbs as a flexible platform for outbreak response, post-exposure prophylaxis, and treatment of patients at high risk of severe orthoflavivirus disease due to compromised health, advanced age, IFN impairment or during pregnancy (Zika).

Although vaccines and antibody-based interventions are central to orthoflavivirus prevention and treatment, they must be integrated with public health measures that reduce exposure at the source. Vector control remains a main strategy of orthoflavivirus prevention and complements vaccines and therapeutics. Traditional mosquito-control strategies include insecticides, larvicides, elimination of standing water, and the use of personal protection measures such as repellents, window screens, and bed nets.³³¹ Novel approaches are being deployed to overcome insecticide resistance and sustainability issues. These include the release of Wolbachia-infected *Aedes* mosquitoes that reduce vector competence; sterile-insect techniques that release irradiated or genetically sterilized males to suppress populations; and gene-drive technologies designed to spread fitness-reducing traits through mosquito populations.³³¹⁻³³³ Control of tick vectors is more challenging because of their complex life cycles and wildlife reservoirs. Current strategies rely on habitat management, targeted acaricide application, and host-directed interventions such as bait boxes or treated deer feeding stations.³³⁴⁻³³⁶ Effective vector management not only reduces human exposure but also limits viral amplification in reservoir hosts, decreasing opportunities for cross-species transmission and emergence of new lineages.

As part of countermeasures against orthoflaviviruses, accurate and timely diagnostics play a critical role in surveillance, outbreak response, and evaluation of vaccine and therapeutic efficacy. Serological confirmation and differentiation of orthoflavivirus infections still rely on assays using authentic viruses, particularly plaque reduction neutralization tests (PRNTs) and related virus neutralization assays.^{337,338} These assays remain the reference standard for serological confirmation because they measure functional neutralizing antibodies directly and are generally more specific than binding assays in the context of antibodies cross-reactivity to multiple antigenically-related, cocirculating viruses.³³⁹⁻³⁴¹ Their use is especially valuable when distinguishing closely related infections or confirming exposure in vaccinated or previously infected individuals.³⁴² However, assays with authentic virus are labor-intensive, relatively slow, difficult to standardize across laboratories, and, for several orthoflaviviruses, may require

higher biosafety containment and advanced expertise, which limits their scalability for large serosurveys, outbreak response, and diagnostics.³⁴³⁻³⁴⁵

To overcome these hurdles and facilitate high-throughput screening, pseudovirus systems such as Reporter Virus Particles (RVPs) are increasingly used. These systems allow neutralization testing in Biosafety Level 2 (BSL-2) settings by using single-cycle infectious particles, thereby enabling safer and more scalable serological analyses.^{164,346,347} Such systems can also support antibody discovery and characterization, although their performance depends on the specific assay design and virus system used.

Looking ahead, because antigenic drift and changing vector distributions continually reshape orthoflavivirus epidemiology, integrated programs combining vaccination, therapeutic monoclonal antibodies and vector control will have to be supported by sustained genomic and serologic surveillance. Monitoring of viral evolution, population immunity-such as through detection of serum neutralizing antibodies to orthoflaviviruses not known to circulate in a given region-will provide early warning of cryptic viral circulation and inform vaccine updates, therapeutics deployment, and overall outbreak preparedness.^{165,321}

3. Aim of the thesis

The overall aim of this thesis is to characterize the human antibody response against orthoflavivirus infections, with a particular focus on mAbs targeting the virus EDIII protein. By defining EDIII epitopes linked with potent virus-specific neutralization, as well as conserved epitopes that mediate cross-neutralization among mosquito- and/or tick-borne orthoflaviviruses, this work seeks to identify structural features that can guide the development of broadly protective antibody-based therapeutics. In parallel, this work investigates the interplay between antibody responses and innate antiviral immunity, including the role of IFN-I responses and the impact of autoantibodies directed against these molecules. Together, the thesis provides insight into mechanisms of protection, disease susceptibility, and immune recognition across medically relevant orthoflaviviruses.

Finally, by documenting serological evidence of orthoflavivirus exposure in regions where certain members have not been previously reported, this thesis highlights the ongoing circulation and geographic expansion of orthoflaviviruses and supports the need for improved surveillance, preparedness, and broadly applicable countermeasures.

4. Results

4.1 Cross-reactive antibodies against tick-borne and mosquito-borne orthoflaviviruses

Human antibodies in Mexico and Brazil

neutralizing tick-borne flaviviruses

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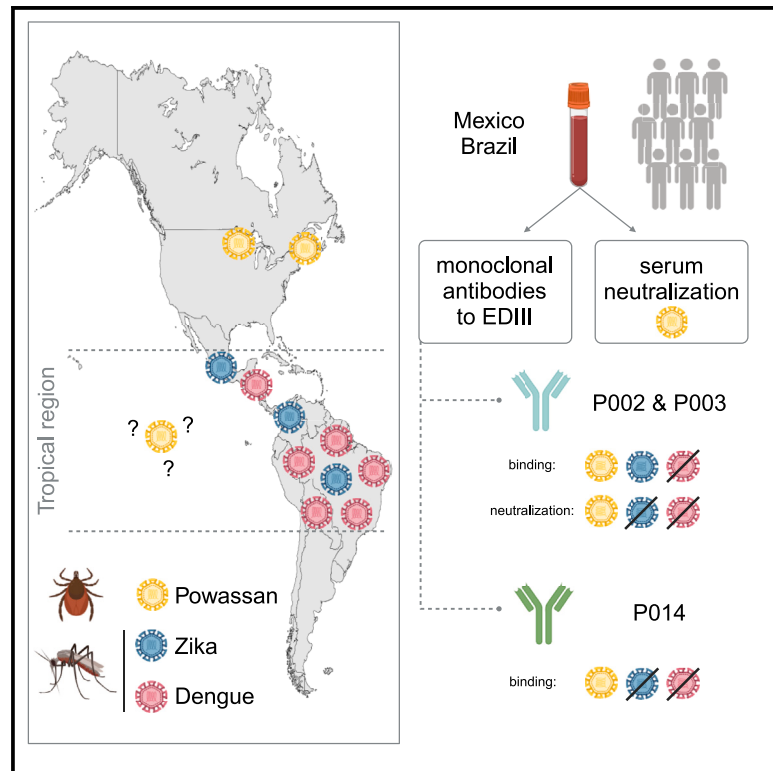
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Contribution: Design, production, purification and characterization of the recombinant EDIII proteins and mAbs for epitope characterization.

Designing and performing experiments, including analysis and interpretation of the results and preparation of the figures (i.e.: ELISA for antibody characterization; mAbs gene amplification, sequencing and cloning; biolayer interferometry assays; computational analysis of the EDIII protein sequences. Figure panels: 2B, 3A-B, 4C-D, 4F-G, 5A, 5C, S3C-E and S4B.

Human antibodies in Mexico and Brazil neutralizing tick-borne flaviviruses

Graphical abstract



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In brief

Cervantes Rincón et al. describe antibody neutralizing activity against Powassan virus from regions of the Americas where this flavivirus transmitted by ticks is not known to circulate. Two monoclonal antibodies to the EDIII (P002 and P003) are broadly cross-reactive against tick- and mosquito-borne flaviviruses.

Highlights

- Sera from Mexico and Brazil neutralize POWV and TBEV, which are not known to circulate there
- Monoclonal antibody P014 binds to POWV but not to endemic flaviviruses
- Monoclonal antibodies P002 and P003 neutralize POWV lineage I
- The P003 epitope is shared across 13 flaviviruses from tick and mosquito serocomplexes



Article

Human antibodies in Mexico and Brazil neutralizing tick-borne flaviviruses

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SUMMARY

Flaviviruses such as dengue virus (DENV), Zika virus (ZIKV), and yellow fever virus (YFV) are spread by mosquitoes and cause human disease and mortality in tropical areas. In contrast, Powassan virus (POWV), which causes severe neurologic illness, is a flavivirus transmitted by ticks in temperate regions of the Northern hemisphere. We find serologic neutralizing activity against POWV in individuals living in Mexico and Brazil. Monoclonal antibodies P002 and P003, which were derived from a resident of Mexico (where POWV is not reported), neutralize POWV lineage I by recognizing an epitope on the virus envelope domain III (EDIII) that is shared with a broad range of tick- and mosquito-borne flaviviruses. Our findings raise the possibility that POWV, or a flavivirus closely related to it, infects humans in the tropics.

INTRODUCTION

Flaviviruses (the term flavivirus is used in the text to refer to members of the genus *Orthoflavivirus*) include at least 30 human pathogens, which are transmitted by diverse arthropod vectors.^{1,2} In contrast to the many mosquito-borne flaviviruses that are endemic to tropical areas, there is only one tick-borne flavivirus known to cause human disease in the Americas: Powassan virus (POWV), which is an emerging concern in the United States and Canada.³ Infection by POWV may cause encephalitis, which can be lethal or lead to persisting neurological symptoms. No vaccine or specific treatment exists for POWV. Although

there are two lineages of POWV, each with distinct enzootic cycles, serological responses to both lineages are reported to be indistinguishable.³

Because flaviviruses are antigenically related, antibodies induced by one flavivirus may bind to others. This complicates serology-based diagnostics when multiple flaviviruses co-circulate in the same geographical region. Moreover, antibodies generated during primary infection by one flavivirus can protect against disease upon secondary infection by the same or a distinct flavivirus but may also enhance the severity of the disease in some cases due to antibody-dependent enhancement (ADE).^{4–6} Tick- and mosquito-borne flaviviruses are phylogenetically and antigenically



divergent, leading to their classification in distinct serocomplexes: POWV is a member of the tick-borne virus complex, which is distinct from the complexes to which dengue virus (DENV), Zika virus (ZIKV), yellow fever virus (YFV), and West Nile virus (WNV) belong.^{7–10}

We unexpectedly discovered human antibodies capable of POWV neutralization from residents of tropical environments in Mexico and Brazil that are hyperendemic for mosquito-borne flaviviruses but where the circulation of POWV or other known tick-borne flaviviruses that infect humans has not been reported to date. POWV-neutralizing, human monoclonal antibodies derived from a resident of Mexico recognize an epitope on the virus envelope domain III (EDIII) that is broadly conserved across mosquito- and tick-transmitted flaviviruses.

RESULTS

POWV can be transmitted by the same species of tick (*Ixodes scapularis*) that transmits *Borrelia* species, including those that cause Lyme disease.³ Thus, to examine whether individuals from the New York metropolitan area might have been exposed to POWV, we examined by enzyme-linked immunosorbent assay (ELISA) serum samples from suspected or confirmed Lyme disease cases ($n = 556$).¹¹ As controls, we included samples collected following a DENV outbreak in Salvador, Brazil, and after a ZIKV outbreak in Santa Maria Mixtequilla, Oaxaca, Mexico ($n = 49$ and $n = 109$, respectively; Figure 1; see STAR Methods). The level of serum immunoglobulin G (IgG) reactivity to the EDIII of POWV (lineage II) was significantly higher in the samples from Mexico and Brazil compared to that of the New York cohort (Figure 1A). A similar result was obtained when measuring the IgG reactivity to the EDIII of tick-borne encephalitis virus (TBEV), a tick-borne flavivirus that causes human disease in Europe and Asia (Figure 1B).¹²

The samples from Mexico and Brazil were collected within a few months of mosquito-borne flavivirus outbreaks, when antibody cross-reactivity is high and specificity of binding is low. Thus, we examined whether the sera binding to POWV and TBEV in ELISA were also neutralizing using luciferase-encoding reporter virus particles (RVPs) expressing the structural proteins of the corresponding flaviviruses (see STAR Methods).¹³ In comparison to the samples from New York, significant POWV RVP neutralization was observed in a fraction of the samples from tropical regions, particularly those originating from Mexico (Figure 1C). The neutralization capacity of TBEV RVPs by the same samples was generally lower than for POWV (Figure 1D). To confirm the results of the screening, we measured the serum antibodies of MEX58, a study participant from whom we were also able to obtain peripheral blood mononuclear cells (PBMCs). The amount of IgG binding to the POWV EDIII was comparable to a sample from an individual vaccinated against TBEV, and the two samples also bound similarly to the TBEV EDIII (Figures 1E and 1F). In contrast, MEX58 displayed selective neutralization of POWV RVPs (half-maximal neutralization titer $[NT_{50}] = 4.9 \times 10^3$) over TBEV (Figures 1G and 1H). Conversely, the TBEV-vaccinated sample was mostly effective against TBEV RVPs ($NT_{50} = 7.6 \times 10^3$; Figures 1G and 1H). The virus-neutralizing capacity of the serum from MEX58 was confirmed with authentic POWV lineage I and lineage II (Figure 1I).

We conclude that individuals living in Mexico and Brazil have sera that neutralize POWV and that although the antibodies of MEX58 recognize the EDIIIs of both POWV and TBEV flaviviruses, they are more effective at neutralizing POWV.

To further characterize POWV antibodies, we used a previously established strategy to identify virus-binding B cells from the PBMCs of MEX58 (Figure 2; see STAR Methods).¹⁴ Single IgG-switched memory B cells binding to the fluorescently labeled POWV EDIII were isolated by flow cytometry, and 31 antibodies were identified by sequencing (Figures 2A and S1; Table S1). Four monoclonal antibodies that were recombinantly expressed bound to the POWV EDIII in ELISA: two representative antibodies from an expanded clone with IGHV1-46 paired with IGKV1-39 (P002 and P003) and two singlets (P014 and P020). With regard to cross-reactivity to endemic viruses, P002, P003, and P020 cross-reacted with the EDIII of ZIKV (and P002 weakly with DENV1 and DENV3), while P014 only recognized POWV (Figure 2B). Thus, monoclonal antibodies were obtained that exhibited narrow specificity to a tick-borne flavivirus (POWV) not known to circulate in the region (i.e., P014), while others were more broadly cross-reactive to endemic viruses (i.e., P002, P003, P020).

Strikingly, in the ELISA, P002 and P003 recognized the EDIII proteins of flaviviruses that can cause human disease and are transmitted by *Ixodes* ticks (POWV, TBEV, Kyasanur forest disease virus, Omsk hemorrhagic fever virus, Louping ill virus, Langkat virus), as well as *Aedes* (ZIKV, YFV) and *Culex* (WNV, Japanese encephalitis virus, Murray Valley encephalitis virus, Saint Louis encephalitis virus, Usutu virus) mosquitoes (Figures 3A and 3B). Thus, P002 and P003 bind to a broad range of flavivirus envelopes. To assess the neutralizing potential of the antibodies, we performed plaque reduction neutralization tests. P002 and P003 neutralized POWV lineage I with a half-maximal inhibitory concentration of 6.8 $\mu\text{g/mL}$. Weak neutralization was detected for WNV, but not for POWV lineage II, ZIKV, or TBEV, even upon incubation at 40°C to increase virus breathing to expose cryptic epitopes (Figures 3C and S2). Thus, although P002 and P003 recognize the EDIIIs of all 5 viruses, they selectively neutralize POWV lineage I and, to a lesser extent, WNV.

To map the P002/P003 epitope, we performed sandwich ELISAs and structural analysis (Figure 4). P002, P003, and previously characterized anti-ZIKV antibodies that recognize the EDIII lateral ridge (Z004, Z021, and Z039) and a non-lateral ridge epitope (Z015)^{14,15} were immobilized on an ELISA surface, and the ZIKV EDIII antigen was captured by the bound antibodies. The binding to the antibody-EDIII complex by His-tagged antibody fragment (Fab) of anti-ZIKV or P002 and P003 antibodies was then evaluated. P002 and P003 recognize similar epitopes on the ZIKV EDIII that do not overlap with those of Z004, Z021, Z039, or Z015 (Figure 4A). Therefore, P002 and P003 recognize the same or similar EDIII epitopes as each other, which does not include the lateral ridge.

Since attempts to crystallize Fab-EDIII complexes were unsuccessful, to uncover the epitope recognized by P002 and P003, we performed nuclear magnetic resonance (NMR) epitope mapping (see STAR Methods and Simonelli et al.,^{17–19} Bardelli et al.,²⁰ Wang et al.,²¹ and Frontzek et al.²²) on P003 Fab binding to the ZIKV EDIII. The ZIKV EDIII was used instead of the POWV EDIII

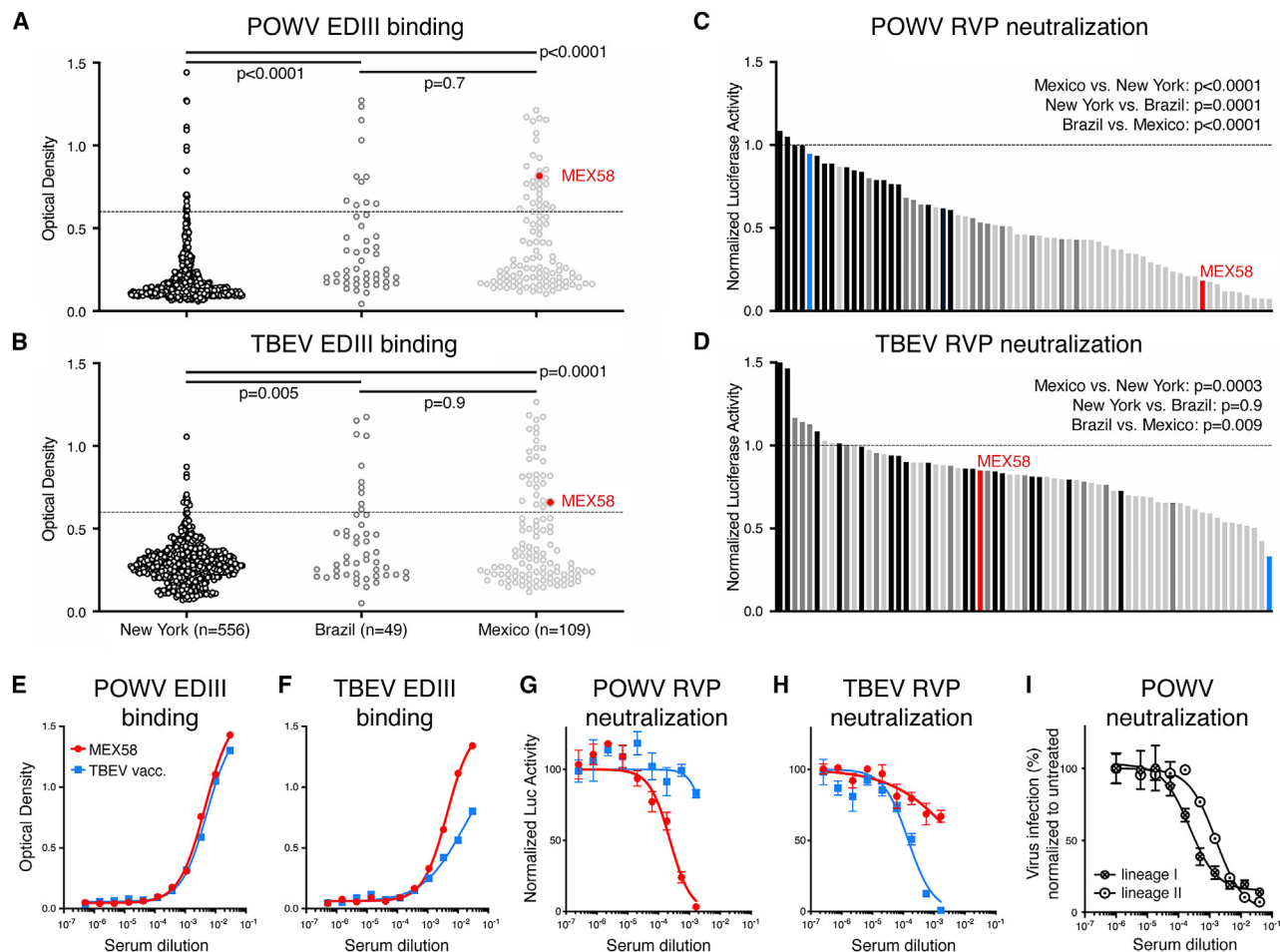


Figure 1. Serum neutralizing activity against POWV in Mexico and Brazil

(A and B) Screening for serum IgG antibodies binding to the EDIII of POWV (lineage II, A) and TBEV (European subtype, B) in ELISA. Each dot represents the IgG reactivity of an individual participant at 1:500 serum dilution (single well). Samples with optical density above 0.6 (dotted lines) were selected for the neutralization screening in (C) and (D).

(C and D) Screening of select sera ($n = 66$) for neutralization of RVPs corresponding to POWV (lineage II, C) and TBEV (European subtype, D). Shown is the rank-ordered luciferase activity relative to “no-serum” control (lower values correspond to higher neutralization). Each bar represents an individual participant sample analyzed at 1:1,200 dilution; average reading of duplicate wells.

(E and F) ELISA IgG binding to POWV (E) and TBEV (F) EDIIIs by serial serum dilutions. Representative of two experiments.

(G and H) Neutralization of POWV (G) and TBEV (H) RVPs by serial serum dilutions. Luciferase (Luc) activity relative to no-serum control. Mean $\pm$ SD of triplicates. Representative of two experiments.

(I) Neutralization of authentic POWV lineage I and lineage II by MEX58 serum using a microscopy-based neutralization assay. One experiment, mean $\pm$ SD of triplicates.

In (A)–(D): black, dark gray, and light gray represent samples from New York, Brazil, and Mexico, respectively. In red is MEX58 and in blue a sample from a TBEV-vaccinated individual. The p values in (A)–(D) were determined with the Mann-Whitney test.

because NMR assignment for the former was available from previous work.²¹ Briefly, the addition of P003 results in changes to the NMR signal of antigen residues due to the direct contact with the antibody or due to allosteric effects. The NMR-derived epitope and cross-competition information was used to validate computational models yielding three-dimensional structures of the P003/ZIKV EDIII complex. The model in best agreement with the experimental data was further subjected to fully atomistic computational molecular dynamics simulations²¹ to increase its precision and accuracy (Figures S3A and S3B). The computational simulations place P003 and its epitope away from the lateral ridge

(Figures 4B and 4C). To further validate the model, we generated mutant ZIKV EDIII proteins with amino acid changes at surface-exposed residues within or near the epitope determined by the NMR data. In agreement with the model, P003 binding to ZIKV EDIII proteins mutated at positions A361, T366, M374, and M375, at the core of the epitope, is greatly reduced, whereas peripheral mutations have a lesser impact (Figures 4C, 4D, and S3C). These results are also consistent with those from the sandwich ELISA (Figure 4A) and the biolayer interferometry assays (Figure S3D) and with the crystal structures of the complexes formed between the EDIII and the Z021- or Z004-related antibody Z006

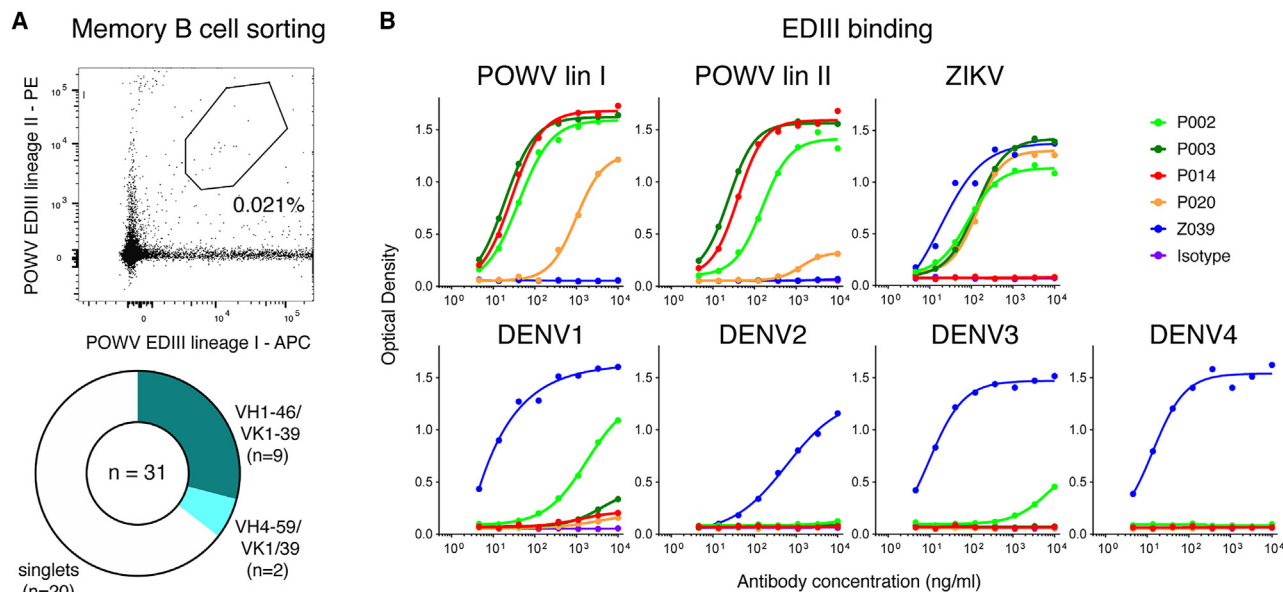


Figure 2. Identification of human monoclonal antibodies binding to POWV EDIII from a resident of Mexico

(A) POWV-EDIII binding antibodies from the memory B cells of MEX58. Top, flow cytometry plot identifying human B cells binding to POWV EDIII proteins corresponding to lineage I and II (gate). The frequency of antigen-binding B cells is shown. Bottom, pie chart showing the distribution of the 31 antibodies. Antibodies that share the same immunoglobulin heavy- and light-chain genes are represented by the colored slices, and singlets are in white.

(B) Monoclonal antibody binding to the EDIIIs of the indicated flaviviruses by ELISA. Z039 is a ZIKV antibody that broadly binds the EDIIIs of all four DENV serotypes and was tested alongside as positive control.¹⁴ Average of two independent experiments.

(Figure 4E; PDB: 6DFI and 5VIG^{14,23}). Additional validation of the model was provided by evaluating POWV lineage I EDIII mutant proteins (Figures 4F, 4G, and S3E).

To explain the breadth of P003 reactivity, we aligned the reference sequences of POWV and other flaviviruses at the EDIII region (Figure S4A). Mapping of the P003 contact residues revealed strong epitope conservation overall (Figures 5A–5C). Consistent with the ELISA results (Figure 2B), DENV1–4 were among the most dissimilar flaviviruses in this analysis. In particular, EDIII residue E329_{ZIKV} is negatively charged in POWV and all other viruses recognized by P003, whereas it loses its negative charge (Q) or gains a positive charge (R, K) in DENV1 or DENV2, DENV3, and DENV4, respectively (Figures 5A–5C). The analysis was extended to all flavivirus envelope sequences deposited in the NCBI Virus database (see STAR Methods), demonstrating high sequence similarity in correspondence of the P003 epitope (Figures S4B and S4C). Finally, K-to-E substitution at position 321 (K321E_{DENV3}, equivalent to E329_{ZIKV}) rescued the binding of P002 and P003 to the DENV3 EDIII, pointing to a central role of this residue for P002/P003 binding to all flaviviruses that were assayed except DENV1–4 (Figure 5C). Therefore, P003 recognizes an epitope on the EDIII that is broadly shared among flaviviruses belonging to several serocomplexes.

DISCUSSION

Symptomatic POWV infection or Powassan disease has been reported in Canada and the northeast of the United States, where POWV is present at variable frequencies in ticks (~3.5%).^{24–26} While searching for antibodies against POWV, we first evaluated

samples of individuals from the New York metropolitan area with suspected or confirmed Lyme disease because POWV is transmitted by the same *Ixodes* ticks that also transmit *Borrelia* species. To our surprise, the New York sera displayed significantly lower POWV-binding and -neutralizing activity compared to control sera from tropical regions of Mexico and Brazil. Central and South America are hyperendemic for flaviviruses transmitted by mosquitoes, but to our knowledge, there are no accounts of tick-borne flaviviruses that infect humans in those regions. Thus, the discovery of POWV-neutralizing sera and the isolation of POWV-neutralizing monoclonal antibodies from tropical regions of the Americas were unexpected.

There are at least two possibilities to explain the development of these POWV antibodies. The first one is cross-reactivity. Residents of tropical regions are recurrently exposed to various DENV serotypes and ZIKV and are at risk of WNV and YFV infection; they also receive the YFV vaccine in some regions. Sequential exposure to different combinations of these mosquito-borne flaviviruses could, in theory, produce antibodies that cross-react with, and even neutralize, phylogenetically more distant viruses of the same genus. This hypothesis clashes with the common view that serum neutralizing activity is flavivirus specific, which is why serologic assays that measure virus neutralization are considered more specific for diagnostic purposes.²⁷ Nonetheless, monoclonal antibodies that broadly cross-react with multiple flaviviruses have been described, even though they more commonly target the virus EDII region, which is more conserved at the sequence level, and not the EDIII that is recognized by the P002 and P003 antibodies.²⁸ Reports of monoclonal antibodies that bind to both tick-borne and mosquito-borne flaviviruses, however, are scarce, which is

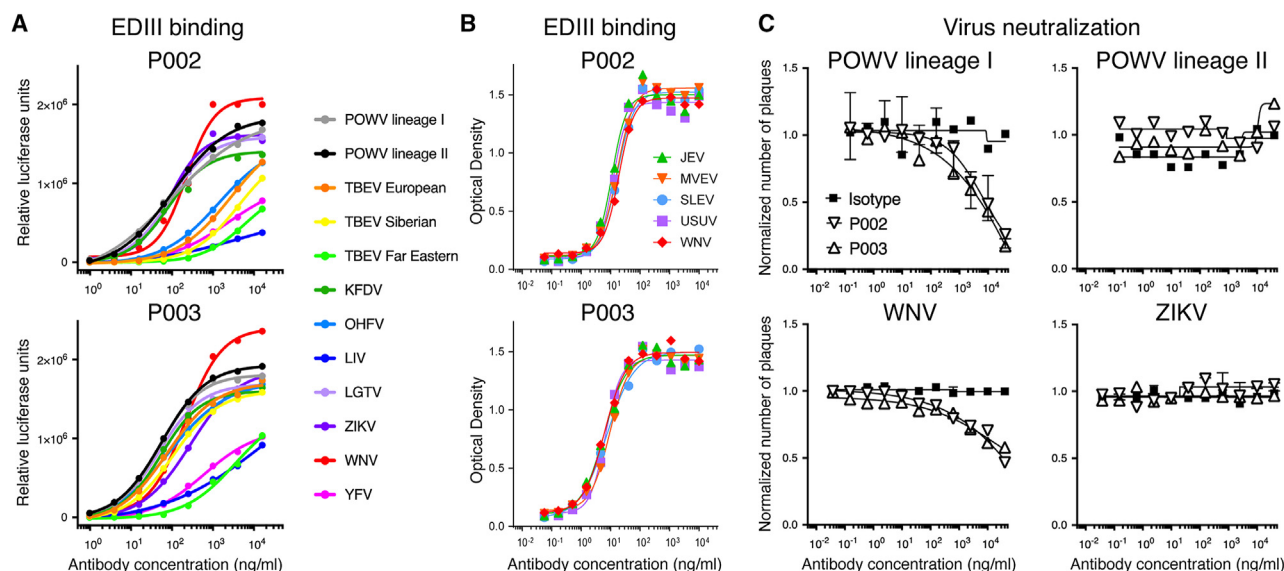


Figure 3. P002 and P003 human monoclonal antibodies are broadly cross-reactive to flaviviruses and neutralize POWV

(A and B) ELISA binding of recombinant human monoclonal IgG antibodies P002 and P003 to the EDIIIs of flaviviruses transmitted by ticks or *Aedes* mosquitoes (A) and *Culex* mosquitoes (B). Representative of two independent experiments.

(C) Neutralization of POWV lineage I by P002 and P003 antibodies. Neutralization was determined with the plaque reduction neutralization test assay and values normalized to no-antibody control. Two experiments (mean $\pm$ SD).

In (A): KFDV, Kyasanur forest disease virus; OHFV, Omsk hemorrhagic fever virus; LIV, Louping ill virus; LGTV, Langat virus. In (B): JEV, Japanese encephalitis virus; MVEV, Murray Valley encephalitis virus; SLEV, Saint Louis encephalitis virus; USUV, Usutu virus.

consistent with the increased antigenic distance between flaviviruses belonging to distinct serocomplexes.^{29,30} We find that P002 and P003 cross-react with a broad range of flaviviruses from at least three serocomplexes, including ZIKV (*Aedes* serocomplex) and WNV (*Culex* serocomplex) but not DENV1–4, which is the most common flavivirus infection in Mexico and Brazil. Remarkably, they fail to effectively neutralize WNV or ZIKV but are effective against POWV instead, a tick-borne flavivirus that the immune system of the individual from which P002 and P003 were derived has presumably not even encountered.

An alternative possibility is the circulation in Mexico and Brazil of POWV or of a flavivirus that is antigenically closely related to it and that infects humans. A public health conference report from 1962 mentions POWV seroreactivity in the Mexican state of Sonora, which is a non-tropical area in the north.³¹ Some tick-borne flaviviruses, such as Kyasanur forest disease virus, which causes hemorrhagic fever in India, are known to infect humans in tropical areas, but they are not known to be present in the Americas.³² Nevertheless, ticks, including the *Ixodes* species that are vectors for POWV in temperate regions, are present in the tropics.³³ Such a flavivirus could easily remain undiscovered if it rarely or never causes human disease or if the infection causes symptoms that are indistinguishable from a common circulating virus such as DENV that is often diagnosed solely based on clinical presentation.

The existence of such a virus could have significant implications. On one hand, circulation of yet another flavivirus in tropical Americas would further complicate diagnostics. On the other, it could potentially induce antibodies that alter the disease of subsequent flavivirus infections due to antibody cross-reactivity and the possibility of disease enhancement (ADE). Further studies

are required to determine the origin of POWV-neutralizing antibodies in individuals in Brazil and Mexico.

Limitations of the study

One limitation of the study is that the infection history of the study participants as well as their demographic information (age, sex/gender, ethnicity) were not available or obtained, and thus the influence (or association) of these factors on the results of the study is not reported. Furthermore, the samples were collected within a few months of outbreaks by mosquito-borne flaviviruses (DENV in Brazil and ZIKV in Mexico): serum antibodies elicited by one flavivirus can bind to others (especially shortly after an outbreak), possibly leading to false positive results in the ELISA. However, the cross-reactivity of binding is not expected to significantly influence the ability of the antibodies to neutralize flaviviruses belonging to distinct serocomplexes.^{29,34–36} The study cannot resolve whether the antibodies neutralizing POWV were induced by a tick-borne flavivirus or by sequential exposure to different combinations of mosquito-borne flaviviruses but prompts further investigations that include the analysis of ticks in these regions of the Americas.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
 - Lead contact
 - Materials availability

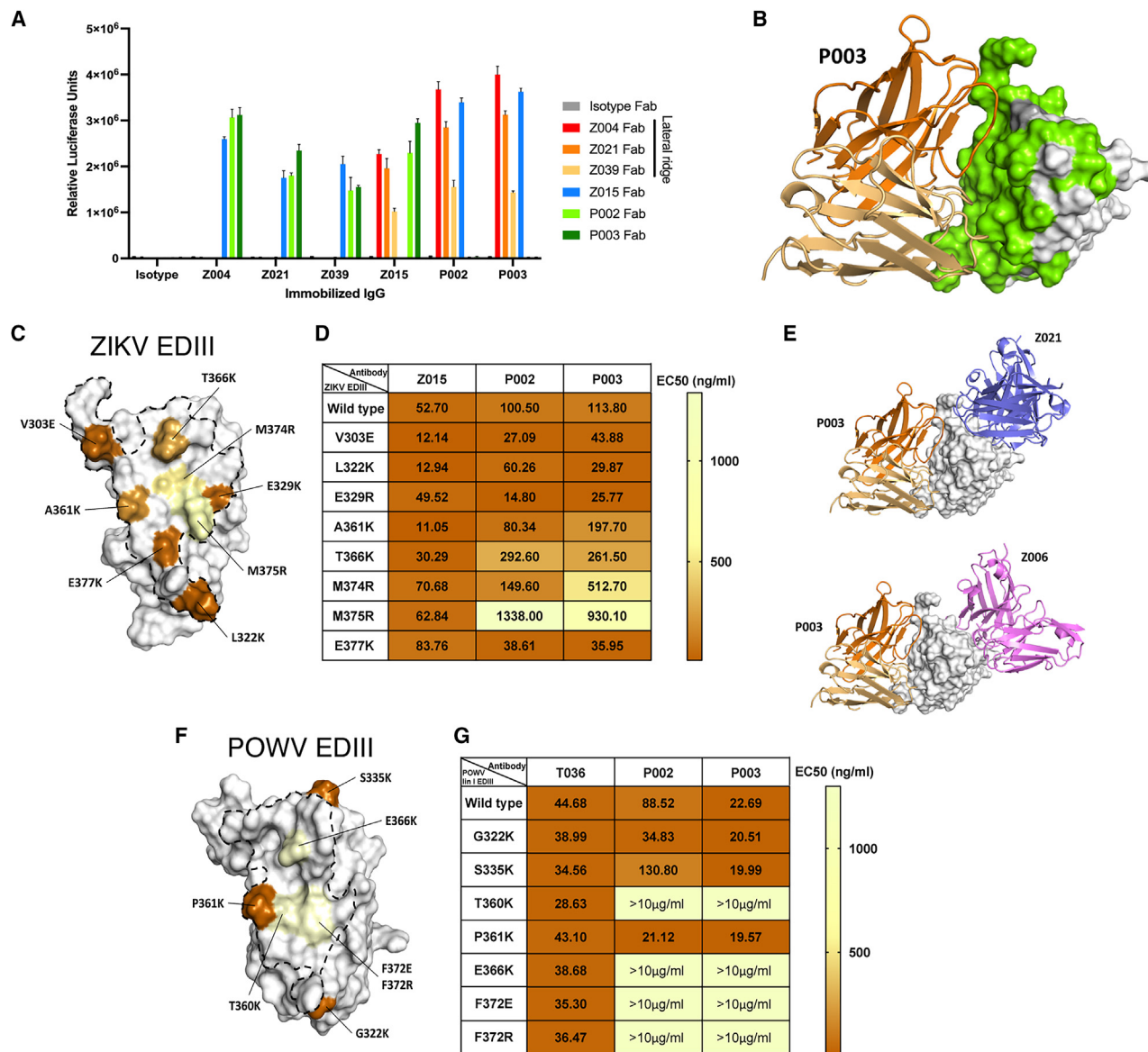


Figure 4. P003 recognizes an epitope away from the EDIII lateral ridge

(A) Sandwich ELISA shows that P002 and P003 recognize an EDIII epitope distinct from previously reported ZIKV antibodies. His-tagged Fabs were assessed for binding to the ZIKV EDIII immobilized by a specific IgG. Binding of the Fab suggests a non-overlapping epitope with the IgG; no binding suggests a similar or overlapping epitope with the IgG. Mean $\pm$ SD of quadruplicates.

(B) Model of the P003-ZIKV EDIII complex from NMR-validated computational simulations. EDIII residues affected by antibody binding are in green on the surface of EDIII (PDB: 5OMZ). P003 is the orange cartoon, with the light chain in lighter tones. See also [Figures S3A](#) and [S3B](#).

(C) Structure of ZIKV EDIII (white) with selected amino acids colored according to the half-maximal effective concentration (EC_{50}) value of P003 binding to the EDIII with the amino acid change shown in (D). Contours indicate the P003 epitope.

(D) Binding of P003 and P002 to ZIKV EDIII mutated at selected epitope residues assessed by ELISA. Summary of EC_{50} binding values. Z015 is a ZIKV antibody binding to a distinct epitope. See also (C) and [Figure S3C](#).

(E) P003 (orange) binds to a different EDIII region than Z021 (blue) or Z006 (pink).

(F and G) Same as in (C) and (D) for validation of the model using POWV (lineage I) EDIII wild-type and mutant proteins. T036 is a TBEV antibody that also binds POWV¹⁶ and recognizes a distinct epitope. See also [Figure S3E](#).

- Data and code availability
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 - Viruses
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- **METHOD DETAILS**
 - Reagents
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 - Sandwich ELISA
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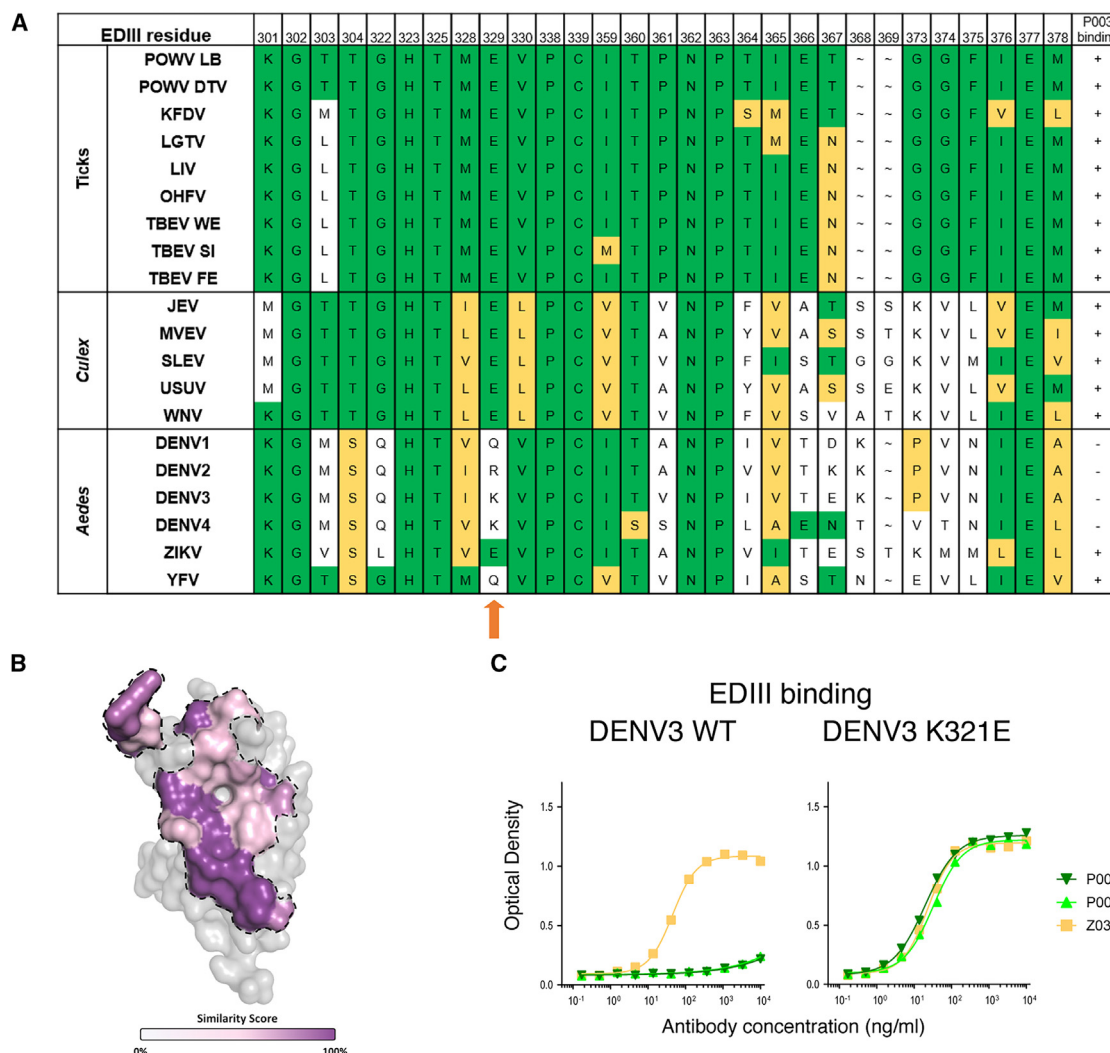


Figure 5. The P003 epitope on the EDIII is highly conserved across flaviviruses except for DENV

(A) Alignment of the P003 contact residues with the sequences of the EDIII proteins used in ELISA (Figures 2 and 3). Highlighted in green are the identical residues and in yellow those that are similar by side-chain functionality to the corresponding residues in POWV. The arrow points to the P003 epitope at position 329 (ZIKV numbering). Related to Figure S4A.

(B) Coldmaps representing on the ZIKV EDIII the frequency of identical or similar residues at the P003 epitope (dashed contour) across the flaviviruses recognized by P003 and indicated in (A).

(C) Rescue of P002 and P003 binding by the K321E substitution in DENV3 (equivalent to E329_{ZIKV}). Average of two independent ELISA experiments.

- Plaque reduction neutralization test (PRNT)
- Microscopy-based neutralization assay
- Isolation of virus-specific B cells
- Antibody sequencing, cloning and production
- Biolayer interferometry
- NMR spectroscopy
- Antibody modeling, docking and MD simulations
- EDIII sequence analysis
- **QUANTIFICATION AND STATISTICAL ANALYSIS**

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.celrep.2024.114298>.

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AUTHOR CONTRIBUTIONS

T.C.R., T.K., J.R.K., L.S., H.-H.H., M.A., A.J., A.P., Y.E.L., A.G., F.G., J.C., B.C., F.B., E.T., and P.S. produced reagents, performed experiments, and analyzed the data; L.S. and S.G.M. performed computational structural and sequence analyses; M.G.R., F.C., A.I.K., B.A.F., S.A.-R., and G.R.-T. provided human samples; C.M.R., M.C.N., P.J.B., D.R., and L.V. supervised portions of the work; J.R.K., M.R.M., and D.F.R. conceived the study, coordinated and supervised the execution, analyzed the data, and wrote the manuscript, with contributions by the other authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-IgG (human) Sheep Polyclonal Antibody HRP	Cytiva	Cat. No: NA933; RRID:AB_772208
Human recombinant antibody 10-1074	Mouquet et al. ³⁷	N/A
Human recombinant antibody Z004, Z015, Z021, Z039	Robbiani et al. ¹⁴	N/A
Human recombinant antibody T036	Agudelo et al. ¹⁶	N/A
Human recombinant antibody hr2.023	Bianchini et al. ³⁸	N/A
PE-Cy TM 7 Mouse anti-human CD20	BD Biosciences	Cat. No: 560735; RRID:AB_1727450
APC Mouse anti-human IgG	BD Biosciences	Cat. No: 562025; RRID:AB_10892809
THE TM His Tag HRP-conjugated	Genscript	Cat. No: A00612; RRID:AB_915573
Bacterial and virus strains		
<i>E.coli</i> BL21(DE3)	New England Biolabs	Cat No: 25271
POWV (lineage I); Byers strain	CDC	N/A
POWV (lineage II); Spooner strain	CDC	N/A
TBEV; Hypr strain	Collection of Arboviruses, Institute of Parasitology, Biology Center of the Czech Academy of Sciences, Cask Budejovice, Czech Republic	N/A
WNV; NY99 strain	Shi et al. ³⁹	
ZIKV; PRVABC59 strain	CDC	N/A
Chemicals, peptides, and recombinant proteins		
Albumin (BSA) Fraction V (pH 7.0)	PanReac AppliChem	Cat. No : A1391
Avicel	FMC BioPolymer	Type RC-581; CAS#9004-34-6
Carboxymethylcellulose	Sigma-Aldrich	Cat. No : C4888
Dimethylsulfoxide (DMSO)	Sigma-Aldrich	Cat. No: 41640
DMEM, high glucose, GlutaMAX TM	Thermo Fisher Scientific	Cat. No: 61965-026
Dulbecco's Phosphate Buffered Saline (PBS)	Sigma-Aldrich	Cat. No: D8537
EDTA solution pH 8.0 (0.5M)	PanReac AppliChem	Cat. No: A3145
Fetal Bovine Serum (FBS)	Gibco	Cat. No: A5256701
Formaldehyde	Fisher	Cat. No: F79-1
L-glutamine	Sigma-Aldrich	Cat. No: G7513
Leibovitz (L-15)	Biosera	Cat. No: LM-L1050
Medium 199	Gibco	Cat. No: 11150059
MEM w/ Earles Salts w/ Glutamine	Biowest	Cat. No: L0415
Methylcellulose	Fisher	Cat. No: M352-500
Naphthalene black solution	Sigma-Aldrich	Cat. No: 195243
Octet 1M Ethanolamine	Sartorius	Cat. No: 18-1071
Opti-MEM	Gibco	Cat. No: 31985062
Pierce TM TMB Substrate Kit	Thermo Fisher Scientific	Cat. No: 34021
Streptavidin APC Conjugate	Thermo Fisher Scientific	Cat. No: 17-4317-82
Streptavidin PE Conjugate	Thermo Fisher Scientific	Cat. No: 12-4317-87
Sulfuric Acid	Sigma-Aldrich	Cat. No: 258105

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
TBEV(WE)-, TBEV(FE)-, TBEV(Si)-, POWV(LB)-, POWV(DTV)-, OHFV-, KFDV-, LIV- and LGTV-EDIII proteins	Agudelo et al. ¹⁶	N/A
Tween®20	Sigma-Aldrich	Cat. No: P7949
ZIKV-, DENV1, DENV2, DENV3, DENV4, YFV(17D)-, WNV-EDIII proteins	Robbiani et al. ¹⁴	N/A
USUV-, JEV-, SLEV-, MVEV-EDIII proteins	This manuscript	N/A
ZIKV-, DENV3-, POWV(LB)-EDIII mutant proteins	This manuscript	N/A

Critical commercial assays

BirA Biotin-Protein Ligase kit	Avidity	Cat. No: BirA500
ExpiFectamine™ CHO Transfection Kit	Gibco	Cat. No: A29129
LS Columns	Miltenyi Biotec	Cat. No: 130-042-401
Maxima H Minus First Strand cDNA Synthesis Kit	Thermo Fisher Scientific	Cat. No: K1651
Mouse anti-human CD19 microbeads	Miltenyi Biotec	Cat. No: 130-050-301
Pan B-cell isolation kit	Miltenyi Biotec	Cat. No: 130-101-638
Phusion High Fidelity DNA Polymerase	New England Biolabs	Cat. No: M0530
QuikChange Multi Site-Directed Mutagenesis Kit	Agilent Technologies	Cat. No: 200514
SuperSignal ELISA Femto Substrate	Thermo Fisher Scientific	Cat. No: 37074

Deposited data

EDIII sequence analysis	This manuscript	Github: https://doi.org/10.5281/zenodo.11125547
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Experimental models: Cell lines

Lenti-X 293T	Clontech	Cat. No: 632180
Expi293F	Thermo Fisher Scientific	Cat. No: A14568
VERO E6	ATCC	Cat. No: CRL-1586
BHK-21	ATCC	Cat. No: CCL-10
Porcine stable (PS) kidney cells	National Cell Culture Collection, National Institute of Public Health, Prague, Czech Republic	N/A
HEK293-6E	ATCC	Cat. No: CRL-1573
Huh-7.5	Blight et al. ⁴⁰	N/A

Oligonucleotides

Primers for antibody cloning by the SLIC method	Robbiani et al. ¹⁴	N/A
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Recombinant DNA

IGγ1-, IGκ or IGλ-expression vectors	Tiller et al. ⁴¹	N/A
pPOWV-DTV-CprME	Agudelo et al. ¹⁶	N/A
pPOWV-LB-CprME	Agudelo et al. ¹⁶	N/A
pTBEV-WE-CprME	Agudelo et al. ¹⁶	N/A
pWNVII-Rep-REN-IB	Laboratory of T.Pierson	N/A
pZIKV/HPF/CprME	Laboratory of T.Pierson	N/A

Software and algorithms

GROMACS	Van Der Spoel et al. ⁴²	N/A
IgBlast	Ye et al. ⁴³	https://www.ncbi.nlm.nih.gov/igblast/
RosettaAntibody	Sircar et al. ⁴⁴	N/A
GraphPad Prism (v.8.4.3)	GraphPad	RRID:SCR_002798
RosettaDock v3.1	Gray et al. ⁴⁵	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Octet® Analysis Studio 12.2	Sartorius	N/A
EDIII sequence analysis	This manuscript	https://github.com/simonemoro/MA2consensus
Biorender	Biorender	https://BioRender.com
Other		
FLUOstar Omega Luminometer	BMG LabTech	N/A
FLUOstar Omega microplate reader	BMG LabTech	N/A
BioTek Cytation 7 Cell Imaging Multimode Reader	Agilent	N/A
Octet® Amine Reactive 2nd Generation (AR2G) Biosensors	Sartorius	Cat No: 18-5092
Octet® R8 System	Sartorius	RRID:SCR_023267

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Davide Robbiani (drobbiani@irb.usi.ch).

Materials availability

Expression plasmids and other materials will be shared upon request; distribution may require a material transfer agreement.

Data and code availability

- No datasets composed of standardized datatypes were generated. Antibody sequencing data are provided in [Table S1](#). Additional data are available upon request from the [lead contact](#).
- All original code developed for EDIII sequence analysis is publicly available, see [key resources table](#).
- Any additional information required to reanalyze the data reported in this work paper is available from the [lead contact](#) upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Viruses

The Byers strain (lineage I) of POWV was obtained from the CDC (Viral Special Pathogens Branch stock 808908) with a recorded passage history of 9 times in suckling mice, 1 time in Vero cells, 1 time in VeroE6 cells and 1 time in Huh-7 cells. A working stock was generated by infection of BHK-21 cells at a multiplicity of infection (MOI) of 0.002 plaque-forming units (PFU)/cell and harvesting of the medium 2 days post infection. RNA from the working stock BHK-21-derived virus was isolated with Trizol according to the manufacturer's recommendations. The genome copies/mL was determined by qRT-PCR as described.⁴⁶ After cDNA synthesis using the Maxima H Minus First Strand cDNA Synthesis kit (Thermo Fisher Scientific), the open reading frame region was PCR amplified in two pieces with Phusion High Fidelity DNA Polymerase (NEB) and primer pairs RU-O-28679 (5'- AACGTGAGTGTGTTGAGAAAAAGAC)/RU-O-28676 (5'- TCCTCTTGTCACATGCCACATGGTGTGTA) and RU-O-28677 (5'- GATGCCATGGGGAACCTGCACTTGAC)/RU-O-28678 (5'- GCGACCAATTTCCCGTCACATGTGTGT). The two fragments were pooled and submitted to the Harvard DNA Core Facility for MiSeq sequencing. Results indicated 3 nucleotide (nt) changes in the consensus sequence compared to the LB strain of POWV (L03436.1): viral nt 3492 T to G, nt 6537 T to C (both synonymous changes), and nt 6260 C to T (threonine to methionine) in NS3. Viral stocks were stored in single use vials at -80°C and were titrated on Huh-7.5 cells by plaque assay (8.95×10^6 PFU/mL; genome/PFU ratio of 70.3). Based on how the neutralization assay was performed, 6,298 genome copies were added in each well.

Lineage II POWV (DTV, Spooner strain) was generated from a 2-plasmid infectious clone system kindly provided by Aaron Brault and the viral genomic RNA generated as described (CDC,^{46,47}). Briefly, plasmids DTVp1 and DTVp2 were digested with BstZ171, ligated at a 1:3 ratio, linearized with NotI and used as a template for RNA transcription using Epicenter T7 RNA polymerase according to the manufacturer's recommendations in the presence of cap analog (NEB). The RNA was electroporated into Huh-7.5 cells and the medium harvested 7 d later to generate a stock. RNA was isolated from the stock, cDNA synthesized, and two amplicons generated by PCR. Primer pairs utilized were RU-O-28675 (5'- GCGTTCTTTTGAACGTGAGTGTGTTGAGAAAAAG)/RU-O-28676 and RU-O-28677/RU-O-28678. The consensus sequence matched the infectious clone sequence. The genome equivalents per mL of stock was determined by qRT-PCR as described.⁴⁶ Viral stocks were stored in single use vials at -80°C and were titrated on Huh-7.5 cells

by plaque assay (3.1×10^7 PFU/mL; genome/PFU ratio of 61.4). Based on how the neutralization assay was performed, 19,108 or 38,217 genome copies were added in each well.

The ZIKV (2015 Puerto Rican PRVABC59 strain) stock was generated from virus originally obtained from the CDC and passaged twice in Huh-7.5 cells as previously described.¹⁴

WNV (NY99 strain) was generated from plasmid pFLWNV essentially as described.³⁹ Briefly, XbaI linearized plasmid template was used for T7 driven *in vitro* RNA transcription using the mMessage mMachine High Yield Capped RNA T7 Transcription kit (ThermoFisher) according to the manufacturer's instructions. After electroporation of the RNA into BHK-21 cells the medium was harvested 2 days later to generate the virus stock.

We used low-passage tick-borne encephalitis virus (TBEV) strain Hypr, originally isolated in 1953 from a 10-year-old child with meningoencephalitis in Brno, Czech Republic (formerly Czechoslovakia).⁴⁸

Cell lines

All mammalian cell lines were grown at 37°C. Human embryonic kidney HEK293-6E cells were cultured in DMEM 10% FBS at 8% CO₂. African green monkey kidney Vero cells and human hepatocyte Huh-7.5 cells⁴⁰ were cultured in DMEM supplemented with 1% non-essential amino acids and 10% FBS at 5% CO₂. Human Lenti-X 293T and hamster kidney BHK-21 cells were cultured in MEM with 7.5% FBS at 5% CO₂. Porcine kidney stable (PS) cells⁴⁹ were grown in Leibovitz (L-15) medium supplemented with fetal bovine serum (3%), a mixture of antibiotics (100 U/ml penicillin and 100 µg/mL streptomycin) and L-glutamine (1%) (Sigma-Aldrich) at 37°C and normal atmosphere.

Human samples

Serum and PBMCs were obtained from consenting participants under protocols approved by the ethical committees of Rockefeller University (DRO-0898), Columbia University (#7683, #5847, #6805), National Institute of Respiratory Diseases, Mexico (C16-16), Yale University (IRB HIC 1603017508), and the Oswaldo Cruz Foundation, Brazilian Ministry of Health (CAAE 63343516.1.0000.5028). Samples from individuals from the New York metropolitan area were collected through the Columbia University Lyme and Tickborne-Diseases Research Center in the period from 2014 to 2019¹¹; those from Mexico were obtained in the Spring/Summer of 2016 from residents of Santa Maria Mixtequilla, within 3 months from the beginning of the ZIKV outbreak; those from Brazil were collected from a cohort of residents from an urban informal settlement which was followed in 2010, within 3 months from the beginning of a large DENV outbreak.⁵⁰ Serum aliquots were inactivated at 56°C for 1 h and then stored at 4°C until use. Analysis of the influence (or association) of infection history, age, sex/gender, ethnicity and other demographic factors was not performed because this information was not available or obtained. PBMCs of donor MEX58 were purified by gradient centrifugation with Ficoll and frozen in liquid nitrogen in 90% heat-inactivated FBS and 10% DMSO. All samples were shipped to The Rockefeller University, where the analyses were performed.

METHOD DETAILS

Reagents

EDIII proteins

Envelope Domain III proteins were expressed by BL21(DE3) *E. coli* as described previously^{14,51} and were either untagged or Histidine- and Avi-tagged at the C terminus (for cell isolation experiments, Figure 2A). Plasmids for expressing the EDIII of ZIKV (strain – PRVABC59), DENV1, DENV2, DENV3, DENV4, YFV (strain – 17D), WNV (strain – NY99), TBEV (Western European, Siberian, and Far Eastern subtypes), POWV lineage I (strain – LB), POWV lineage II, DTV (strain – Spooner), OHFV, KFDV, LIV, and LGTV were previously described and their corresponding GenBank numbers are indicated in Figure S4A.^{14,16,51} DNA sequences encoding the EDIIIs corresponding to USUV residues 298–401 (Vienna 2001; GenBank: NC_006551.1), JEV residues 298–401 (SA-14; GenBank: M55506.1), SLEV residues 297–400 (Parton; GenBank: EF158070.1) and MVEV residues 299–402 (MK6684; GenBank: KF751869.1) were codon-optimized for *E. coli* expression and cloned into an expression vector.

Mutant ZIKV, DENV3 and POWV lineage I EDIII proteins were produced using plasmids modified by site-directed mutagenesis (QuickChange multikit, Agilent Technologies #200514). For cell sorting experiments, the POWV-LB (lineage I) and POWV-DTV (lineage II) EDIII proteins were biotinylated at the Avi-tag with the BirA Biotin-Protein Ligase kit (Avidity) according to the manufacturer's instructions.

Reporter virus particles

RVPs were generated in Lenti-X 293T cells as reported in detail¹⁴ by co-transfection of a plasmid encoding the nonstructural proteins of a WNV luciferase-expressing replicon (pWNVII-Rep-REN-IB;¹³) with a plasmid expressing the structural proteins of the flavivirus of interest. After incubation for 48–96 h, supernatants were harvested, filtered (0.22 µm), aliquoted and frozen at –80°C. Plasmids to express the structural proteins corresponding to TBEV (strain – Neudoerfl, Western European subtype) and POWV lineage II, DTV were reported previously.¹⁶

Antibodies

The human recombinant IgG1 antibodies Z004, Z015, Z021, Z039, T036, and the isotype control antibodies (hr2.023 and 10–1074), were previously reported.^{14,16,37,38}

ELISA assays

Binding of diluted serum IgG or recombinant IgG antibodies to flavivirus EDIII proteins was determined by ELISA as previously described.¹⁴ Briefly, 250ng of protein in PBS were coated per well of a 96-well plate, overnight, and blocking was with 1% BSA 0.1mM EDTA in PBS-T (PBS with 0.05% Tween 20). Secondary HRP-conjugated anti-IgG (Jackson ImmunoResearch; 109-036-088) was used for detection, followed by TMB substrate (Thermo Fisher Scientific; 34021). The reaction was stopped using 1 M sulfuric acid and the plates read at 450 nm with FLUOstar Omega microplate reader (BMG LabTech). The ELISA data of the New York group in Figure 1A correspond to the re-analysis of previously reported data.¹¹ Experiments in Figures 4D and 4G were performed with 384-well plates, with volumes adjusted accordingly and antigen at 5 μ g/mL. For screening, serum samples were diluted at 1:500 with PBS-T. For recombinant antibodies, binding is expressed as the half-maximal effective concentration (EC_{50}) and calculated with Prism software (GraphPad).

Sandwich ELISA

Monoclonal IgG (5 μ g/mL) was adsorbed overnight at 4°C in a Nunc MaxiSorp 384-well ELISA plate. The ELISA plate was blocked with 3% BSA in TBS-T (TRIS-buffered saline with 0.1% Tween 20), then 5 μ g/mL ZIKV EDIII was added and incubated for 60–90 min at room temperature. The plate was washed with TBS-T to remove excess antigen, then 5 μ g/mL Fab was added and incubated for 3 h at room temperature. Bound His-tagged Fab was detected using HRP-conjugated THE His Tag Antibody (Genscript Cat # A00612; 1 h, room temperature) and developed with SuperSignal ELISA Femto Substrate (Thermo Fisher). Relative light units (RLU) were plotted as a function of IgG-Fab pair.

Neutralization of luciferase-encoding RVPs

Neutralization of luciferase-encoding RVPs. Neutralization was performed in triplicates as previously described.¹⁴ Briefly, dilutions of serum or antibodies were incubated with a fixed concentration of RVPs for 1 h at 37°C and then added to Huh-7.5 cells for 24 h. After lysis, renilla luciferase activity was read with a FLUOstar Omega luminometer (BMG LabTech). The neutralizing capacity was determined by the fraction of luciferase signal relative to signal of RVPs incubated without antibodies (“no-serum” control). NT_{50} (half-maximal neutralization titer) indicates the reciprocal of the serum dilution resulting in 50% neutralization over control and was determined with GraphPad Prism.

Plaque reduction neutralization test (PRNT)

ZIKV PRNT was performed essentially as described previously¹⁴ with minor modifications. Briefly, 1:1 mixtures of a constant amount of ZIKV in OptiMEM with serial dilutions of antibody diluted in BA-1 (Medium 199 supplemented with 1% BSA, 0.05 M Tris, 100 units/mL penicillin, 100 μ g/mL streptomycin) were prepared and incubated for 1 h at 37°C. 0.4 mL of each antibody dilution/virus mixture was then applied to Vero cells seeded in 6-well plates 24 h earlier at 2×10^5 cells/well. After incubation at 37°C for 1.5 h with rocking, 3 mL of 1.2% Avicel in DMEM was added as a semi-solid overlay. After 4 days the wells were fixed with 3.5% formaldehyde and stained with crystal violet to enumerate plaques. WNV PRNT was performed similarly, using 1% methylcellulose in DMEM as the semi-solid overlay, with fixation after 2 days. POWV PRNT assays were conducted similarly with the following modifications: Huh-7.5 cells (3.2×10^5 per well seeded 24 h prior) were used as target cells. For lineage I (strain - Byers) 1.2% Avicel in DMEM was used as the overlay, with fixation 4 days later, while for lineage II (strain - Spooner) 1% methylcellulose in DMEM was used with fixation 6 days later. For TBEV PRNT, the antibodies were diluted in the 96-well plate and incubated with 50 PFU of TBEV per well at 37°C for 1.5 h in the final volume of 100 μ L. Following the incubation, the mixture was transferred into the 24-well plate, and 300 μ L of PS cells were added at a density of 1.8×10^5 per well. After 3–4 h at 37°C, the attached cells were overlaid with 1.5% carboxymethylcellulose (w/vol) in the L-15 medium and incubated at 37°C for 5 days. Then, the cell monolayers were stained with naphthalene black solution (0.1% of naphthol blue-black, 6% glacial acetic acid, and 1.36% sodium acetate), and the plaques were counted. The experiment was performed in triplicates. $PRNT_{50}$ values were determined in GraphPad Prism as the concentration of antibody resulting in a 50% reduction in plaque number compared to no antibody controls.

Microscopy-based neutralization assay

The day prior to infection Huh-7.5 cells were seeded at 1×10^4 cells/well into 96-well plates. Serum (MEX58) and antibodies (P002, P003) were serially diluted in OptiMEM supplemented with 1% BSA. The diluted samples were mixed with a constant amount of ZIKV, POWV lineage I or POWV lineage II. The starting concentration for the serum was a 1:25 dilution and for the antibodies 40 μ g/mL. The serum-virus mix was incubated for 60 min at 37°C, while the antibody-virus mix was incubated for 1 h at 37°C and at 40°C. Both the serum-virus-mix and antibody-virus-mix were then directly applied to Huh-7.5 cells ($MOI_{ZIKV} = 1 \times 10^4$ PFU/well, $MOI_{POWV I} = 7.5 \times 10^3$ PFU/well, $MOI_{POWV II} = 2.5 \times 10^4$ PFU/well). Cells were incubated for 24 h, fixed by adding an equal volume of 7% formaldehyde to the wells, and followed by permeabilization with 0.1% Triton X-100 for 10 min. After extensive washing, cells were incubated for 1 h at room temperature with blocking solution of 5% goat serum in PBS (catalog no. 005-000-121; Jackson ImmunoResearch). A mouse monoclonal anti-flavivirus envelope protein antibody (catalog no. NBP25270902; FisherScientific) was added to ZIKV-infected cells at a 1:500 dilution in blocking solution and incubated at 4°C overnight. A chicken monoclonal anti-Langkat virus NS3 protein antibody (kindly provided by Sonja Best, NIH) was added to POWV-infected cells at a 1:2,000 dilution in blocking solution and incubated at 4°C

overnight. Goat anti-mouse Alexa Fluor 488 (catalog no. A-11001; Life Technologies) and goat anti-chicken Alexa Fluor 488 (catalog no. A-11039; Life Technologies) secondary antibodies were used at a 1:2,500 dilution. Nuclei were stained with Hoechst 33342 (catalog no. 62249; FisherScientific) at a 1 μ g/mL concentration. Images were acquired with a fluorescence microscope and analyzed using a BioTek Cytation 7 Cell Imaging Multimode Reader (Agilent, Santa Clara, CA).

Isolation of virus-specific B cells

PBMCs were resuscitated and prepared for sorting by flow cytometry similar to previously described.¹⁴ Briefly, B cells were positively enriched on magnetic columns with CD19 microbeads (Miltenyi) prior to staining with anti-CD20, anti-IgG, and fluorescently labeled POWV-EDIII baits. The fluorescent baits were produced by incubating 3 μ g of biotinylated POWV-LB-EDIII with streptavidin-allophycocyanin (APC) or 3 μ g of biotinylated POWV-DTV-EDIII with streptavidin-phycoerythrin (PE) for at least 1 h in the dark at 4°C. After washing, single CD20⁺IgG⁺POWV-DTV-EDIII⁺POWV-LB-EDIII⁺ memory B cells were sorted as single cells into individual wells of 96 well plates using a FACSARIAII cytometer.¹⁴

Antibody sequencing, cloning and production

Antibody genes were amplified by PCR from cDNA of single cells and sequenced as described previously.¹⁴ V(D)J gene assignment was with IgBlast. P002 and P003 were cloned into human IgG1 immunoglobulin expression vectors by the SLIC method⁵² and the variable regions of the expressed antibodies correspond to the following sequences:

P002 heavy chain:

EVQLVQSGAEVKKPGASVKISCETSGYSFSTYYIHVWRQAPGQGLEWMGVISPGSGSTNYAQKFQGRVTMTGDTATSTVYMELSSLRS
EDTAVYFCARVKSRMTLIRGVQFDPWGQGTLTIVSS;

P002 light chain:

DIQMTQSPSSLSASVGDRTITCRASQSISNLFNWHYHQKPGQAPKLLIYAASLTQSGVPSRFSGSGSETDFTLTISLQPEDATYFCQQ
SYITPITFGHGTRLEIK;

P003 heavy chain:

EVQLVQSGAELKKPGASVKVSKASGNTFTNDYLHWVRQAPGQGLEWMGIINPNSGSTYYAQRFGRLTMTDTSTSKIYMELSGLR
SEDTAVYYCARGQGDKTLVRGVILVYWGQGTIVTVSS;

P003 light chain:

DIQMTQSPSSLSASVGDRTISCRASQSISTYLNWFQKPGKAPKVLVYSASSLQSGIPSRFSGSGSGTDFTLTISLQPEDFATYYCQQ
SYITPLTFGGGTKVEIK.

P014 heavy chain:

EVQLVQSGAEVKKPGETLRISCKGSGYSFTDYWVWRQTPEKGLEWMGRINPRDSYTNYPSPFQGHVTLADKSVSTAYLQWSSLK
ASDTAIYYCARQYLMTSAGLPFDSWGQGTIVTVSS;

P014 light chain:

DIQMTQSPSSLSASVGDRTITCRASQSVSIYLNWYQKPGKAPKLLVYTASNLTQSGVSSRFSGSGSGTEFTLTISLEPEDFATYYCQQ
SYLSPTFGGGTKVEIK;

P020 heavy chain:

QVQLVQSGAEVKKPGASVKVSKASGDTFTSNHMHVWRQAPGQGLEWMGIINPGSGSTNYAQKFQGRVTMTSDTSTSTVYMELSS
LRSEDTAVYYCARGGLKIAVAGYFDYWGQGTIVTVSS;

P020 light chain:

DIVMTQSPDSLAMSLGERATINCKSSQSVLYSSSNKNSLAWYQKPGQPPLLIYWASTRESGVPDRFSGSGSGTDFTLTISLQAEDV
AVYYCQQYYSTPLTFGGGTKVEIK.

Antibodies were produced in HEK293-6E. For Fabs, the heavy chain variable regions were cloned using the Gibson method to insert the VH into a Fab heavy chain expression vector encoding a C-terminal His tag, and expression was in Expi293 cells using Expifectamine transfection reagent (ThermoFisher Scientific).

Biolayer interferometry

Biolayer interferometry (BLI) assay was performed on an Octet instrument (Sartorius Octet R8 System) operating at 30°C with continuous agitation at 700 rpm with AR2G Biosensors following the manufacturer's protocol 'classical sandwich assay'. In a brief overview, biosensors were hydrated with distilled water 10 min prior to the experiment. To activate sensors, they were immersed for 5 min in a mixture of EDC and s-NHS (20 mM and 10 mM, respectively). Subsequently, to immobilize the first antibody (P003), sensors were immersed for 15 min in a solution of 150 nM diluted in sodium acetate (10 mM, pH 4.5). Biosensors quenching was achieved by immersing sensors for 5 min in Octet 1M Ethanolamine at pH 8.5. The baseline was established by immersing biosensors in PBS for 3 min. For antigen association, biosensors were exposed to a solution containing 100 nM EDIII protein for 15 min. For epitope competition, sensors were immersed for 15 min with the competing antibodies (Z015, Z021 and P003 as a negative control) at 150 nM. The assay was concluded with a 10-min dissociation step in PBS. Processed data were acquired using Octet Analysis Studio 12.2 software and plotted with GraphPad Prism.

NMR spectroscopy

Spectra were recorded on a Bruker Avance 700 MHz NMR spectrometer at 300 K. NMR epitope mapping was performed as previously described.^{17,20} Briefly, overlay of ¹⁵N-HSQC spectra of labeled ZIKV EDIII (H/PF/2013; BMRB code: 34167) free or bound to P003 Fab allowed identification of EDIII residues whose NMR signal changed upon complex formation, indicating that they were affected by P003 binding. Changes were identified by manual inspection and by the Chemical Shift Perturbation (CSP), $CSP = ((\Delta\delta_H)^2 + (\Delta\delta_N/10)^2)^{1/2}$. The concentration used for the NMR mapping experiment was 500 μ M of [¹⁵N]–labeled DIII in 20 mM sodium phosphate, 50 mM NaCl, pH 6.0. Perdeuterated (nominally 70%) ²H, ¹⁵N DIII samples were used for NMR epitope mapping with an EDIII:P003 Fab ratio of 1:1.1.

Antibody modeling, docking and MD simulations

The P003 variable fragment was modeled according to the canonical structure method with the program RosettaAntibody⁴⁴ as previously described.⁵³ For ZIKV EDIII, the NMR calculated structure of DIII (PDB: 5OMZ) was used for docking. Docking P003 Fab model on ZIKV EDIII was performed using RosettaDock v3.1 as previously described.¹⁷ Among the thousands of computationally generated complexes, those in better agreement with NMR experimental data were selected and further refined by molecular dynamics (MD). Selected models were then subjected to a 350 ns MD simulations to adjust the local geometry and verify that the structure was energetically stable. MD was performed with GROMACS.⁴² The system was initially set up and equilibrated through standard MD protocols: proteins were centered in a triclinic box, 0.2 nm from the edge, filled with SPCE water model and 0.15 m Na+Cl-ions using the AMBER99SB-ILDN protein force field. Energy minimization was performed in order to let the ions achieve a stable conformation. Temperature and pressure equilibration steps, respectively at 310 K and 1 Bar, of 100 ps each were completed before performing the full MD simulations with the above-mentioned force field. MD trajectory files were analyzed after removal of Periodic Boundary Conditions. The stability of each simulated complex was verified by root-mean-square deviation and visual analysis.

EDIII sequence analysis

To obtain the EDIII consensus sequence for each of the flaviviruses of interest (Figure S4B), we retrieved from the NCBI Virus database (<https://www.ncbi.nlm.nih.gov/labs/virus/vssi/#/>; September 8, 2023) 56546 annotated E protein sequences by selecting the terms: envelope protein, envelope glycoprotein, envelope, envelope glycoprotein E, envelope protein, envelope protein 1, envelope protein E, partial envelope protein gene for polyprotein, polyprotein, polypeptide, envelope E, truncated polyprotein. We then used BLASTP to align the sequences to the reference ZIKV EDIII and kept those with at least 97% coverage. MAFFT v7.505⁵⁴ was next used to perform the multiple alignment of all filtered sequences to each other. Sequences with ambiguous or missing information (J, B, Z, X) in the region of interest were discarded. Next, the sequences were separated to the flavivirus that they belong to. We finally obtained for each flavivirus of interest the consensus sequences at the EDIII using a script developed in house (<https://github.com/simonemoro/MA2consensus>).⁵⁵

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical tests were as indicated throughout in the figures or their legends. The analyses were performed with Prism software (version 8.4.3; GraphPad), unless otherwise noted.

Supplemental information

Human antibodies in Mexico and Brazil

neutralizing tick-borne flaviviruses

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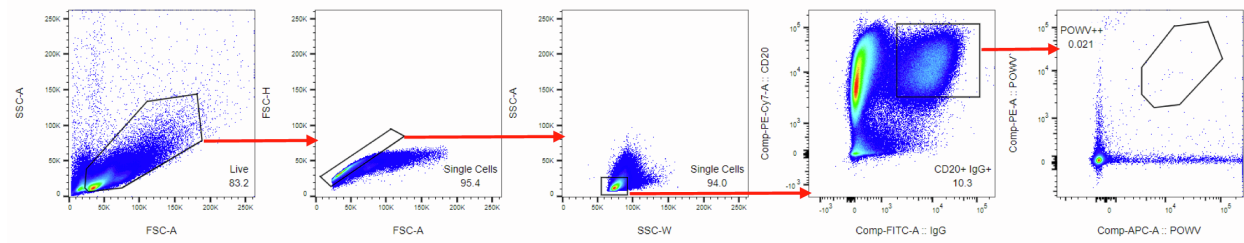


Figure S1. Gating strategy for sorting POWV-EDIII-specific B cells by flow cytometry (related to Figure 2)

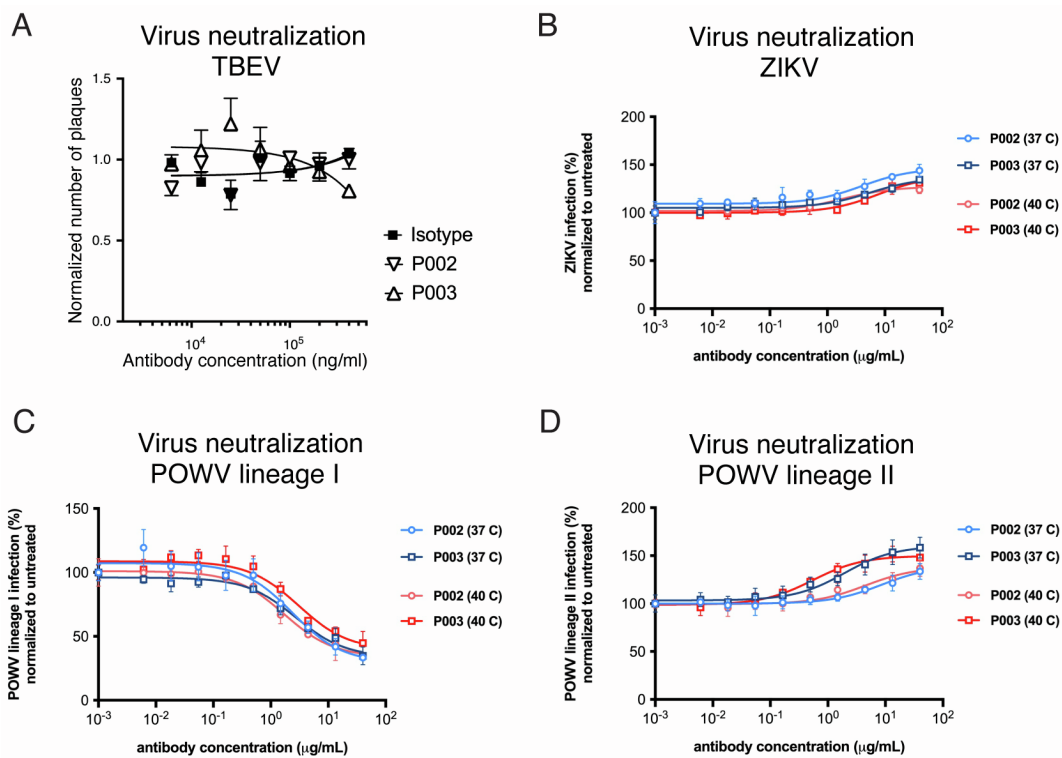


Figure S2. Lack of TBEV neutralization and effects of 40°C incubation (related to Figure 3)

(A) Lack of TBEV neutralization by P002 and P003, as determined by PRNT. Two independent experiments (mean $\pm$ SD).

(B-D) Incubation at 40°C to favor virus breathing does not alter the ability of P002 and P003 to neutralize the indicated flaviviruses, as determined by a microscopy-based neutralization assay. One experiment, mean $\pm$ SD of triplicates.

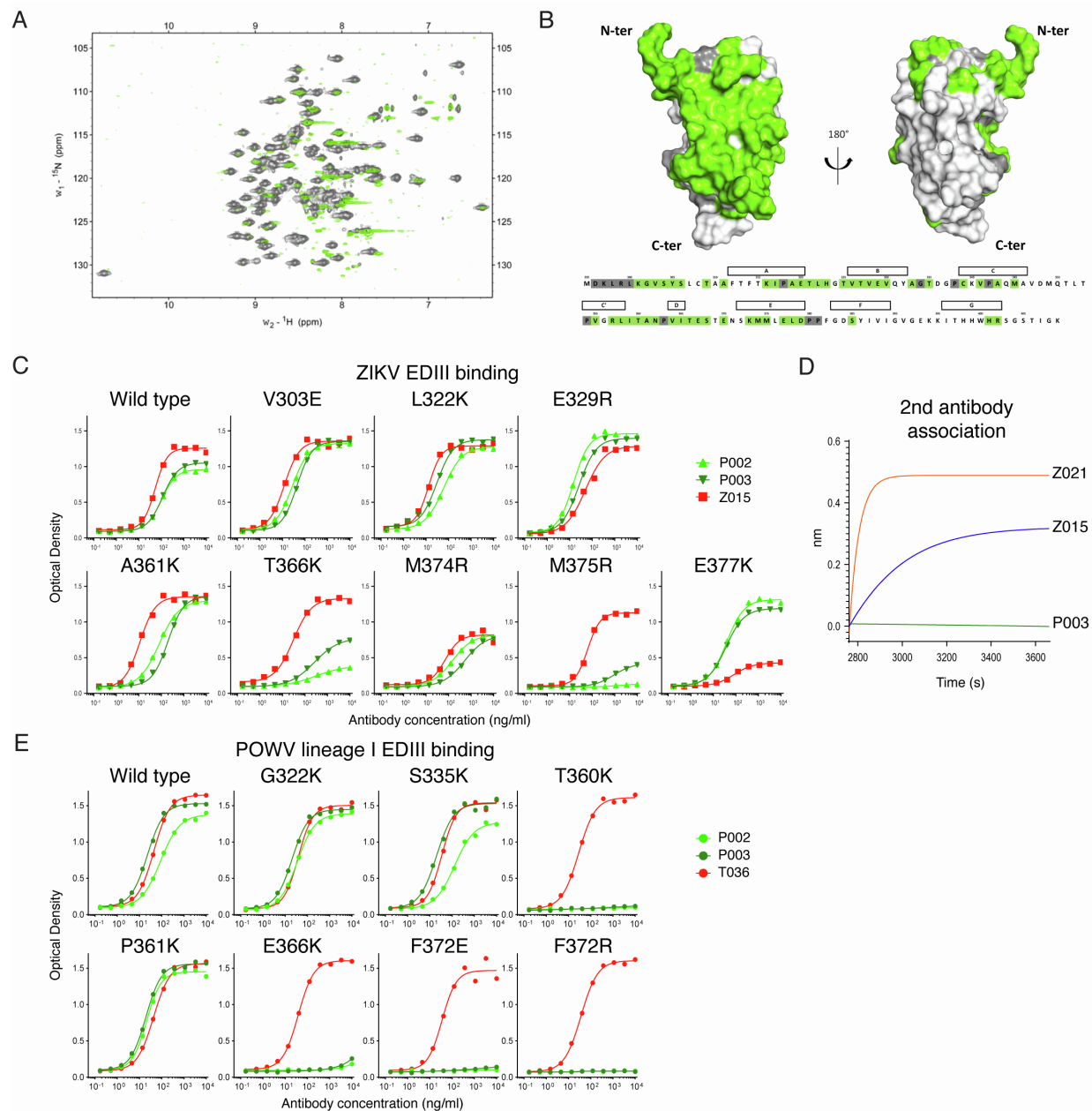


Figure S3. NMR informs on the P003 epitope on EDIII (related to Figure 4)

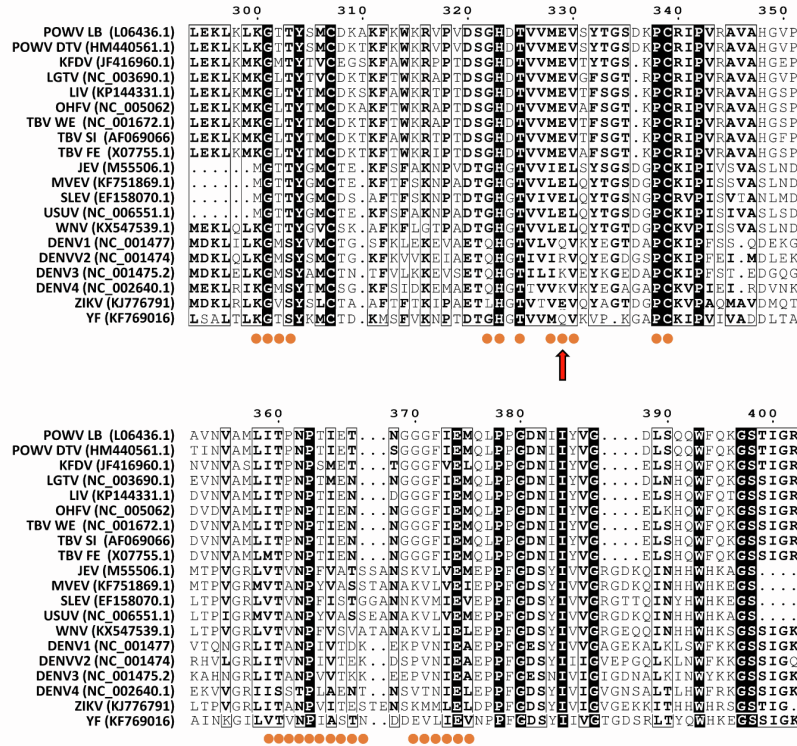
(A) Comparison of the NMR signal of EDIII (grey) with its complex with P003 Fab (green) identifies residues affected by antibody binding.

(B) Residues affected by antibody binding in NMR epitope mapping are shown in green on the surface of ZIKV EDIII (top, two opposite faces are shown; PDB ID: 5OMZ) and on the EDIII sequence (bottom). Residues in grey have no NMR information.

(C and E) Binding of P003 to ZIKV and POWV (lineage I) EDIII mutated at epitope residues, assessed by ELISA. Related to Fig. 4D and 4G. Average of two independent experiments.

(D) Biolayer interferometry confirms binding of P003 to an EDIII epitope that is different from those of antibodies Z015 and Z021. Z021 and Z015 (but not P003 used as negative control), bind to ZIKV EDIII immobilized by P003.

A



B

EDIII residue		301	302	303	304	322	323	325	328	329	330	338	339	359	360	361	362	363	364	365	366	367	368	369	373	374	375	376	377	378	P003 binding
Ticks	POWV	K	G	T	T	G	H	T	M	E	V	P	C	I	T	P	N	P	T	I	E	T	-	-	G	G	F	I	E	M	+
	KFDV	K	G	M	T	G	H	T	M	E	V	P	C	I	T	P	N	P	S	M	E	T	-	-	G	G	F	V	E	L	+
	LGTV	K	G	L	T	G	H	T	M	E	V	P	C	I	T	P	N	P	T	M	E	N	-	-	G	G	F	I	E	M	+
	LIV	K	G	L	T	G	H	T	M	E	V	P	C	I	T	P	N	P	T	I	E	N	-	-	G	G	F	I	E	M	+
	OHFV	K	G	L	T	G	H	T	M	E	V	P	C	I	T	P	N	P	T	M	E	N	-	-	G	G	F	I	E	M	+
	TBEV	K	G	L	T	G	H	T	M	E	V	P	C	I	T	P	N	P	T	I	E	N	-	-	G	G	F	I	E	M	+
Culex	JEV	K	G	T	T	G	H	T	I	E	L	P	C	V	T	V	N	P	F	V	A	T	S	S	K	V	L	V	E	M	+
	MVEV	K	G	T	T	G	H	T	L	E	L	P	C	V	T	A	N	P	Y	V	A	S	S	T	K	V	L	V	E	I	+
	SLEV	K	G	T	T	G	H	T	V	E	L	P	C	V	T	V	N	P	F	I	S	T	G	G	K	V	M	I	E	V	+
	USUV	K	G	T	T	G	H	T	L	E	L	P	C	V	T	A	N	P	Y	V	A	S	S	E	K	V	L	V	E	M	+
	WNV	K	G	T	T	G	H	T	L	E	L	P	C	V	T	V	N	P	F	V	S	V	A	T	K	V	L	I	E	L	+
	WNV	K	G	T	T	G	H	T	L	E	L	P	C	V	T	V	N	P	F	V	S	V	A	T	K	V	L	I	E	L	+
Aedes	DENV1	K	G	M	S	Q	H	T	V	Q	I	P	C	I	T	A	N	P	I	V	T	E	-	-	P	V	N	I	E	A	-
	DENV2	K	G	M	S	Q	H	T	I	R	V	P	C	I	T	V	N	P	I	V	T	E	-	-	P	V	N	I	E	A	-
	DENV3	K	G	M	S	Q	H	T	I	K	V	P	C	I	T	A	N	P	V	V	T	K	-	-	P	V	N	I	E	A	-
	DENV4	K	G	M	S	Q	H	T	V	K	V	P	C	I	S	T	P	F	A	E	N	-	-	-	V	T	N	I	E	L	-
	YFV	K	G	T	S	G	H	T	M	Q	V	P	C	V	T	V	N	P	I	A	S	T	-	-	E	V	L	L	E	V	+
	ZIKV	K	G	V	S	L	H	T	V	E	V	P	C	I	T	A	N	P	V	I	T	E	S	T	K	M	M	L	E	L	+

C

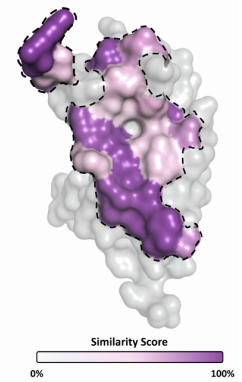


Figure S4. The P003 epitope on the EDIII is highly conserved across flaviviruses (related to Figure 5)

(A) Alignment of the EDIII sequences of flaviviruses known to cause human disease. Dots indicate P003 contact residues, and arrow points to P003 epitope at position 329 (ZIKV numbering). Related to Figure 5A.

(B) Alignment of the P003 contact residues with the EDIII of other human-infecting flaviviruses. Similar to Figure 5A, but based on the consensus sequence of each indicated flavivirus (see STAR Methods). The residue numbering is based on ZIKV EDIII.

(C) Coldmaps representing on the POWV EDIII the frequency of identical residues at the P003 epitope across the flaviviruses recognized by P003. Similar to Figure 5B, but based on the consensus sequence of each indicated flavivirus; related to panel B.

4.2 Human antibodies neutralizing the Japanese encephalitis serocomplex

Analysis of West Nile disease convalescents identifies human monoclonal antibodies protective against West Nile and related orthoflaviviruses

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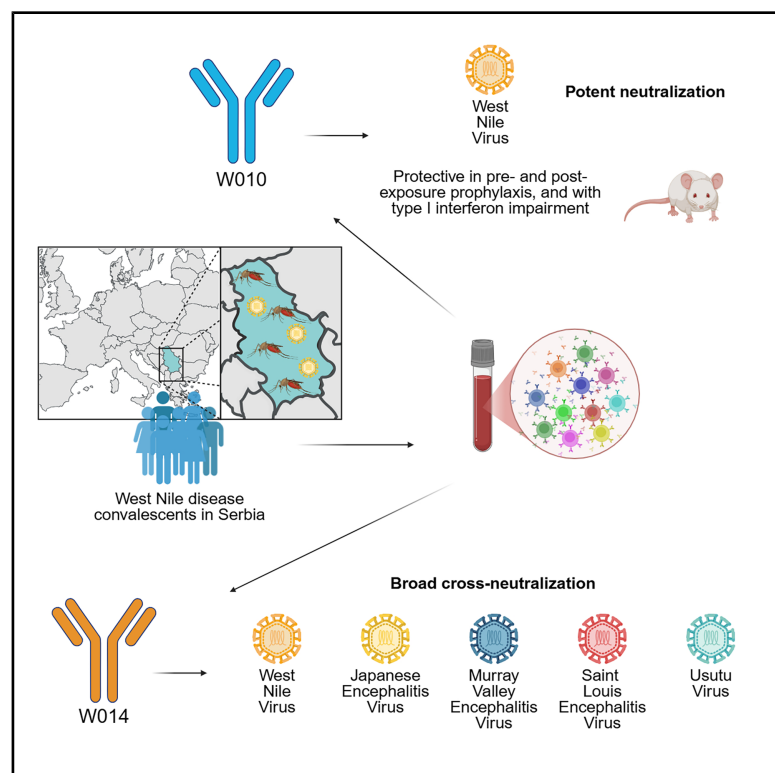
Status: Published in Immunity (2026), <https://doi.org/10.1016/j.immuni.2026.05.013>

Contribution: Memory B cell isolation; design, production, purification and characterization of reagents such as recombinant EDIII proteins, RVPs, and mAbs for epitope characterization.

Designing and performing experiments, including analysis and interpretation of the results and preparation of the figures (i.e.: processing of human samples; isolation and characterization of memory B cells by FACS; ELISA for antibody and serum characterization; mAbs gene amplification, sequencing and cloning; neutralization assays of RVPs; SPR assays. Figure panels: 1A-CC, 2A-B, 3A, 4A, 6A, S1D-P, S2A-E, S3D, S4A-B, S5D-E, S6A-B. Writing and revision of the manuscript with input from all coauthors.

Analysis of West Nile disease convalescents identifies human monoclonal antibodies protective against West Nile and related orthoflaviviruses

Graphical abstract



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In brief

No specific prophylaxis or treatment exists for West Nile virus (WNV) or related orthoflavivirus infections. Cervantes Rincón et al. characterize human antibody responses to WNV and identify protective epitopes on the virus envelope protein. A potent monoclonal antibody protects mice even when interferon signaling is compromised, while another neutralizes related encephalitic orthoflaviviruses. Both represent promising candidates for antibody-based therapeutic development.

Highlights

- Neutralizing IFN-I autoantibodies do not impair antibody responses to WNV
- Human antibodies W010 and W014 target unique epitopes on the WNV EDIII protein
- W010 protects mice both pre- and post-exposure, even with impaired IFN signaling
- W014 is protective and broadly neutralizes multiple encephalitic orthoflaviviruses

Article

Analysis of West Nile disease convalescents identifies human monoclonal antibodies protective against West Nile and related orthoflaviviruses

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SUMMARY

West Nile virus (WNV) is a mosquito-borne pathogen of global concern that can cause fatal neuroinvasive disease, and no specific prophylaxis or treatment exists for infections by WNV and most related orthoflaviviruses. Here, we isolated and characterized antibodies from WNV convalescent individuals and report that neutralizing autoantibodies against type I interferons did not impair antiviral antibody development. Among the monoclonal antibodies with potent neutralizing activity against WNV that were identified, W010 targeted a distinct epitope within the envelope protein domain III (EDIII) and conferred both pre- and post-exposure protection in a murine WNV model, even when interferon signaling was impaired. A second protective antibody, W014, exhibited broad cross-neutralization of other pathogenic orthoflavivirus members, including Japanese encephalitis virus, Murray Valley encephalitis virus, Saint Louis encephalitis virus, and Usutu virus. These findings identify key neutralizing epitopes on WNV EDIII and provide candidates for the development of antibody-based interventions against encephalitic orthoflavivirus infections.

INTRODUCTION

West Nile virus (WNV; *Orthoflavivirus nilense*) is one of many orthoflaviviruses (genus *Orthoflavivirus*) transmitted by *Culex* spp. mosquitoes that are responsible for emerging infectious dis-

eases globally, including fatal encephalitis.^{1–4} While most human WNV infections are asymptomatic, an estimated 20%–40% result in mild disease known as West Nile fever (WNF) and approximately 1% progress to neuroinvasive disease (WNV), which can be lethal.^{5–8} The reasons for the difference in disease

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trajectory upon WNV infection are multifactorial; established risk factors include advanced age and C-C chemokine receptor type 5 (CCR5) polymorphisms.^{9–11} In addition, emerging evidence indicates that the presence of autoantibodies neutralizing type I interferons (IFN-Is: IFN- α and/or IFN- ω) may also impact disease upon infection by WNV and other orthoflaviviruses.^{8,12–16}

WNV is globally endemic and remains the primary cause of mosquito-borne illness in the United States. Since the large outbreak of 2018, it has also become an increasing public health concern in Europe.^{17–20} Projections indicate that the incidence of WND will continue to rise and that the virus may expand into new regions, likely driven by climate change and the associated spread of mosquito vectors.²¹ Despite representing a considerable public health threat, there are no specific prophylactic means or treatments available against WNV-related human diseases.²²

Monoclonal antibodies have demonstrated clinical efficacy against a growing number of infectious diseases, particularly when administered early after the exposure or the onset of symptoms.^{23–25} Although no monoclonal antibody has yet been approved for the prevention or treatment of orthoflavivirus infections, several candidates against yellow fever, dengue, Zika, and tick-borne encephalitis are currently in clinical development.^{26–28} For WND, a humanized mouse-derived monoclonal antibody has progressed to clinical development.²⁹ Of note, transfer of polyclonal immunoglobulins with relatively high titers of anti-WNV antibody failed to show efficacy in humans when administered during late stages of severe disease.³⁰

Similar to other members of the *Orthoflavivirus* genus, WNV displays a single envelope (E) protein on its surface that mediates viral entry.^{5,31} The E protein comprises three domains (I, II, and III), with E domain III (EDIII) forming an immunoglobulin-like fold that plays a key role in attachment to host cells.^{4,32} Antibodies against EDIII have been identified as potent neutralizers of various orthoflaviviruses, including those transmitted to humans by *Aedes* spp. mosquitoes and ticks.^{32–39} While monoclonal antibodies targeting the WNV EDIII have been previously described,^{40,41} these were either of mouse origin or derived from individuals not selected for high serum neutralizing activity.

Although some antibodies showed neutralizing activity against WNV infection, they lacked cross-reactivity with serologically related orthoflaviviruses, such as Japanese encephalitis virus (JEV), Murray valley encephalitis virus (MVEV), Saint Louis encephalitis virus (SLEV), or Usutu virus (USUV), all of which are capable of causing severe, and sometimes fatal, human disease.^{3,42,43}

Here, we report the discovery and characterization of human monoclonal antibodies that potently neutralize WNV, some of which also exhibit cross-neutralizing activity against other mosquito-borne orthoflaviviruses. We identify and characterize the epitope of 4 antibodies, including W010, a highly potent monoclonal antibody that confers both pre- and post-exposure protection against WNV in mice, and W014, which demonstrates broad neutralizing activity against related orthoflaviviruses.

RESULTS

West Nile disease convalescents exhibit distinct antiviral antibody and autoantibody profiles

Seventy-two study participants with suspected WND or WNF were enrolled during 2022 and 2023 in Serbia, and their serum collected during hospitalization (Figures S1A and S1B; Table S1). Serially diluted samples were tested by the enzyme-linked immunosorbent assay (ELISA) for the presence of immunoglobulin G (IgG) antibodies targeting the EDIII protein of WNV (WNV_{EDIII}; Figure 1A). As expected, based on previous studies of the antibody response to other orthoflaviviruses,^{34,44,45} the levels of IgG antibodies to WNV_{EDIII} varied widely.

To assess the virus neutralizing activity of serum, samples were evaluated using an improved WNV reporter virus particle (RVP) system with NanoLuc luciferase (WNV_{RVP}; see STAR Methods; Figures S1C and 1B). Thirty-six individuals with high serum neutralizing activity in the screening were selected for analysis by serial serum dilution (Figure 1C). Half-maximal neutralizing titers (NT₅₀) were variable, with no significant correlation between anti-WNV_{EDIII} IgG (measured as area under the curve [AUC] of ELISA) and WNV_{RVP} NT₅₀ values (Figure S1D). Furthermore, no significant correlation or difference was noted

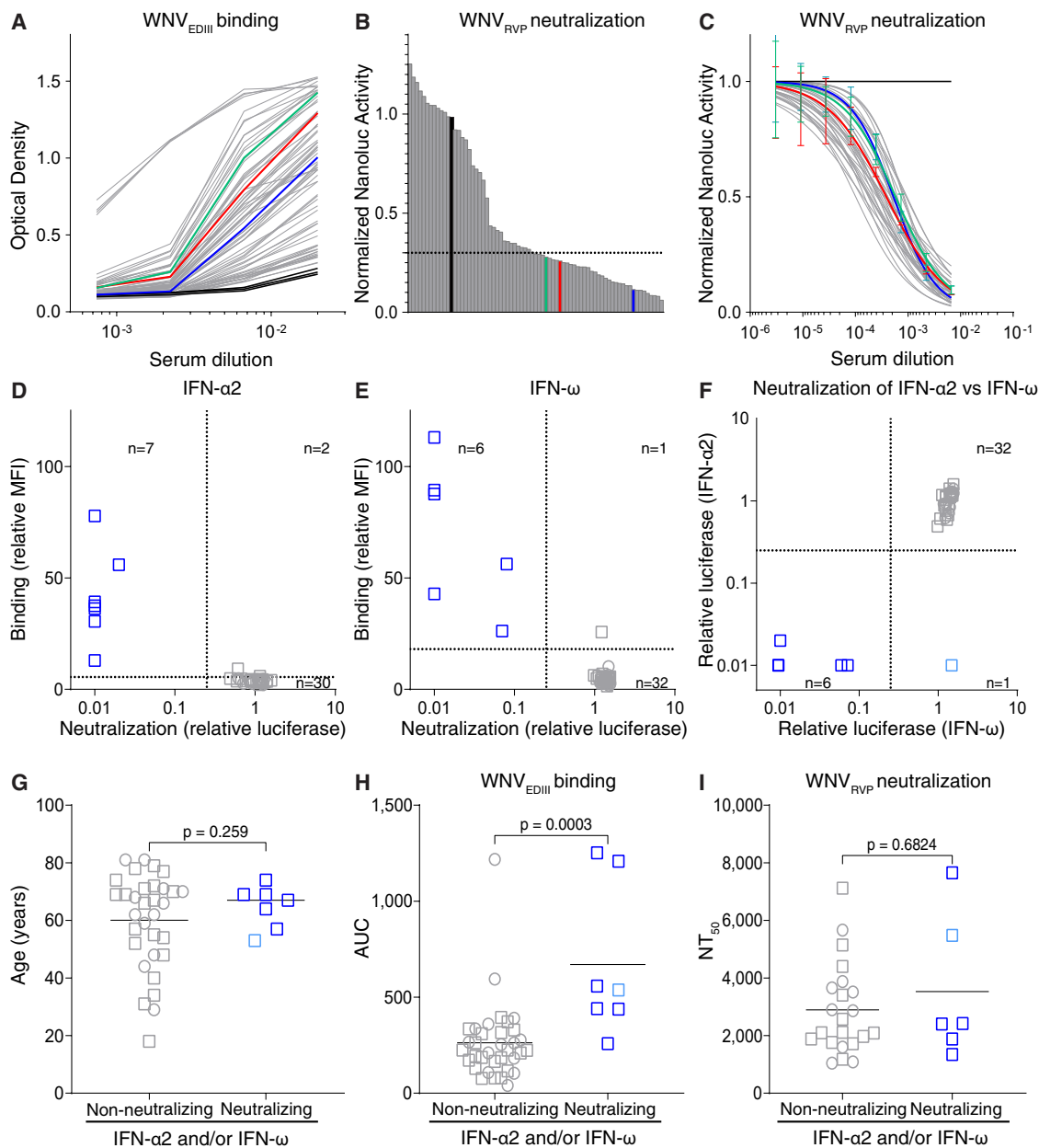


Figure 1. Individuals hospitalized for WND/WNF exhibit distinct serum antibody and autoantibody profiles

(A) IgG binding to WNV_{EDIII} by serial serum dilution. ELISA curves for samples from the 72 WND/WNF-suspected individuals and 3 orthoflavivirus naive controls (black) are shown. The average of two independent experiments is shown.

(B) Serum neutralization screening with WNV_{RVP}. Shown is the rank-ordered NanoLuc activity relative to no-serum control for the 72 WND/WNF-suspected individuals and 1 orthoflavivirus naive control (black); lower values correspond to higher neutralization. Samples with relative NanoLuc signal below 0.3 (dotted line) were selected for neutralization curves in (C). Each bar represents an individual participant sample analyzed at 1:100 dilution. Average signal of triplicate wells from a single experiment.

(C) Neutralization of WNV_{RVP} by serial serum dilution. Shown is the NanoLuc activity relative to no-serum control for 36 WND/WNF cases and 1 orthoflavivirus naive control (black). Mean $\pm$ SD of triplicates. Representative of two independent experiments.

(D–F) Serum autoantibodies binding to and neutralizing IFN-α2 and IFN-ω. Plots compare the ability of serum IgG to bind, and of serum to neutralize, IFN-α2 (D) and IFN-ω (E) (n = 39; only samples for which sufficient serum was available were assayed). The comparison of IFN-α2 and IFN-ω neutralization is shown in (F). Binding is shown as relative mean fluorescence intensity (MFI) and neutralization as relative ISG15-promoter driven luciferase signal compared with no serum control. The dotted lines indicate the threshold for positivity of the assay.^{46,47} Representative of 2 independent experiments.

(G) Age and gender distribution of the study participants with or without IFN-α2 and/or IFN-ω neutralizing autoantibodies. Welch's test.

(legend continued on next page)

in IgG to WNV_{EDIII} (AUC) or WNV_{RVP} neutralization when these features were examined against demographic characteristics, days of hospitalization, or survival, with the exception of a weakly significant positive correlation between IgG to WNV_{EDIII} (AUC) and age, and lower NT₅₀ values for individuals who succumbed to the disease (Figures S1E–S1N).

Serum autoantibodies neutralizing IFN- α and/or IFN- ω have been recently identified as a predisposing factor for WND.⁸ To establish their presence in the cohort and possible association with distinct WNV serologic features, we used a multiplexed bead-based assay to measure IgG autoantibodies binding to IFN- α 2 or IFN- ω ^{46,48} and a recently developed highly sensitive biological assay to determine IFN- α and/or IFN- ω neutralization.⁴⁹ Ten out of 39 individuals with WND (26%) that were analyzed showed detectable serum IgGs targeting IFN- α 2 and/or IFN- ω , and seven of these reactive sera (18%) could neutralize low IFN- α 2 (0.01 ng/mL) and/or low IFN- ω (0.02 ng/mL) doses (Figures 1D–1F). These frequencies represented a significantly higher prevalence than in the general population, as shown by comparison with blood donors and in recent reports^{48,50} (Figures S1O and S1P). The individuals with neutralizing IFN- α and/or IFN- ω autoantibodies were all male and on average slightly older than individuals without these autoantibodies (64.7 versus 60 years) (Figure 1G). Notably, the presence of autoantibodies neutralizing IFN- α and/or IFN- ω did not adversely impact the serologic response to WNV. On the contrary, individuals with these autoantibodies exhibited a significantly increased IgG response to the WNV_{EDIII} protein, while their WNV_{RVP} NT₅₀ values were comparable to those of individuals without such autoantibodies (Figures 1H and 1I). We conclude that the development of antiviral antibodies in individuals hospitalized due to WNV infection is not impaired by the presence of autoantibodies against IFN- α and/or IFN- ω .

Human WNV_{EDIII} antibodies display distinct binding and neutralizing features

To characterize anti-WNV antibodies, we isolated by fluorescence-activated cell sorting (FACS) WNV_{EDIII}-specific B cells from the peripheral blood mononuclear cells (PBMCs) of three convalescent participants with high WNV_{RVP} neutralizing activity (NT₅₀ values ranging between 1,717 and 2,567) and no detectable autoantibodies against IFN- α and/or IFN- ω in serum (Figures 1C, S2A, and S2B). In total, 107 antibody heavy and light-chain gene pairs (89 IgG, 14 IgM, and 4 IgA) were amplified by RT-PCR from individually purified memory B cells and sequenced. Clonally expanded antibody sequences were found in all 3 participants (Figure S2C), and the complementarity-determining region 3 (CDR3) length, as well as the overall number of antibody V gene somatic hypermutations, were similar to previous reports (Figure S2D).^{32,34,51}

Forty-four antibodies (including at least one representative for most of the expanded clones) were expressed recombinantly as IgG1 molecules (Table S2). The half-maximal effective concen-

tration (EC₅₀) values for binding to the WNV_{EDIII} in ELISA was obtained for 32 of them (72.7%) and, consistent with the high similarity at the EDIII between lineage I and II of WNV (the two lineages responsible for most human disease),^{5,19} their binding to the corresponding EDIII was very similar (Figures 2A, 2B, and S2E; Table S3). To determine whether, in addition to WNV, the monoclonal antibodies were reactive to the EDIII of related orthoflaviviruses of the Japanese encephalitis (JE) serocomplex, we produced the EDIII proteins corresponding to JEV, MVEV, SLEV, and USUV for use in ELISA. Some of the antibodies displayed cross-reactivity, including W014 (JEV, MVEV, SLEV, and USUV), W011 (JEV, MVEV, and USUV), and W051 (MVEV and SLEV) (Figure 2A; Table S3). For most of the antibodies, no binding was noted to the EDIII of orthoflaviviruses transmitted by ticks (tick-borne encephalitis virus [TBEV], Powassan virus, Kyasanur forest disease virus [KFDV], Langat virus, Louping-ill virus [LIV], and Omsk hemorrhagic fever virus [OHFV]) or *Aedes* spp. mosquitoes (four serotypes of dengue virus [DENV1–4], yellow fever virus, and Zika virus [ZIKV]), with the exception of W006 (LIV), W023 (ZIKV), and W030 (DENV3, DENV4 and ZIKV) (data not shown). Thus, EDIII antibodies elicited by WNV infection are either specific to WNV or react more broadly with related orthoflaviviruses.

Low-dose W010 protects mice even when administered 5 days after WNV challenge

When evaluated for neutralizing activity, 15 of the 44 antibodies (34%) showed robust WNV_{RVP} neutralization (half-maximal inhibitory concentration [IC₅₀] values below 10 μ g/mL) and two outstanding antibodies were identified: W010 and W049 (IC₅₀ values of 3.7 and 2.0 ng/mL, respectively), both of which outperformed the humanized mouse-derived antibody E16²⁹ (Figure 3A; Table S3). Select antibodies were further tested against authentic WNV lineage I and II by two complementary virus neutralization assays, which confirmed the antibodies' neutralizing capacity and identified W010 as the most potent against both lineages (IC₅₀ values: 4.8 ng/mL for lineage I and 3.8 ng/mL for lineage II when measuring the cytopathic effect; Figures 3B and S3A).

To assess whether W010 protects against disease, we first conducted pre- and post-exposure prophylaxis experiments with BALB/c mice (Figures 3C and S3B). Ten of 12 control mice (83%) that received isotype antibody 24 h prior to lethal challenge with 10⁴ plaque-forming units (PFUs) of WNV lineage II succumbed to the infection by day 10. In contrast, all mice treated with a low dose of W010 (30 μ g/mouse) at either 1 day before, or 1 or 3 days after infection, were protected from disease ($p < 0.0001$ for each group compared with isotype control). Notably, low-dose W010 was also significantly protective when administered 5 days post-infection ($p = 0.0018$).

Next, to mimic the high-risk situation of individuals with autoantibodies to IFN- α and/or IFN- ω , we evaluated the protective effect of W010 in mice conditioned with antibodies blocking the IFN-I receptor (interferon alpha/beta receptor [IFNAR]; see

(H and I) Serum IgG binding to WNV_{EDIII} and serum neutralization of WNV_{RVP} in study participants with or without IFN- α 2 and/or IFN- ω neutralizing autoantibodies. The two groups are compared with respect to (H) IgG binding to WNV_{EDIII} (area under the curve [AUC] of ELISA) and (I) neutralization of WNV_{RVP} (NT₅₀ values). Horizontal lines indicate the mean. Mann-Whitney test.

In (A)–(C), green, blue and red indicate samples from WNV-infected individuals from which antibodies were derived (Figures S2B and S2C). In (D)–(I), female is circle, male is square, dark blue neutralizes both IFN- α 2 and IFN- ω , light blue neutralizes IFN- α 2 only, and gray does not neutralize either.

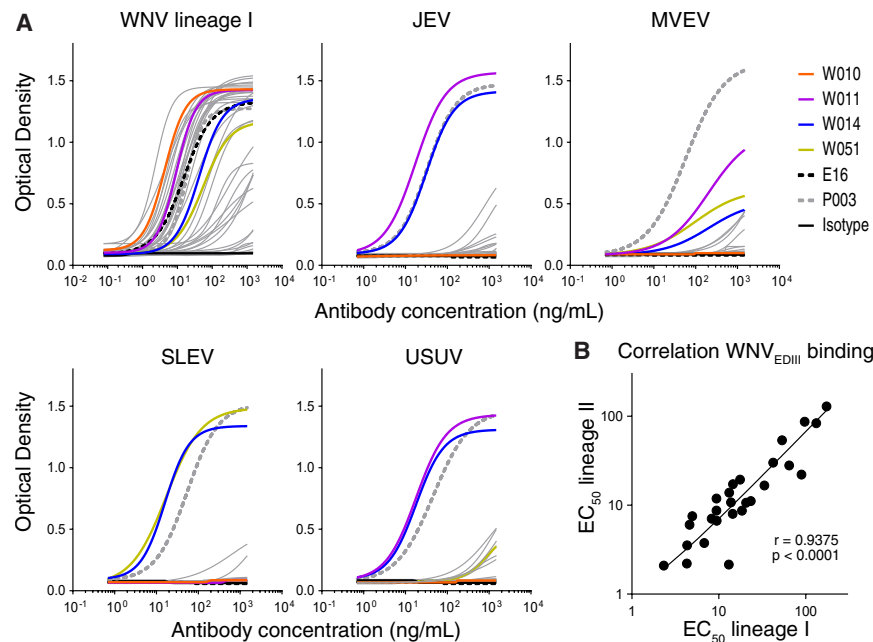


Figure 2. Human monoclonal antibodies isolated from WND/WNF convalescent individuals cross-react with related orthoflaviviruses

(A) Monoclonal antibody binding to the EDIII of WNV and related orthoflaviviruses. ELISA curves show the binding of 44 monoclonal antibodies to the EDIII of the indicated orthoflaviviruses transmitted by *Culex* spp. mosquitoes. Positive control is the broadly reactive antibody P003⁵²; humanized mouse antibody E16⁴¹ is shown for comparison. Representative of two independent experiments.

(B) Correlation of the monoclonal antibodies binding to the EDIII corresponding to WNV lineages I and II. Each dot represents an individual monoclonal antibody EC₅₀ value. Pearson correlation. Related to Figure S2E.

STAR Methods; Figures 3D and S3C). When isotype antibody was administered on the day of WNV infection, all 12 mice succumbed by day 11. In contrast, 58% and 100% of mice receiving W010 on the day of infection or 1 day post-infection, respectively, survived ($n = 12$ and $p = 0.0001$ for both compared with isotype control). W010 was not polyreactive, displayed an average half-life of 104 h in FcγR/FcRn-humanized mice⁵³ and showed a melting temperature of 79.6°C (Figures S3D–S3F). Altogether, these results demonstrate the protective efficacy of W010 even in the context of impaired IFN-I signaling, supporting its potential for prevention and therapy against WNV also in individuals harboring IFN-I autoantibodies.

Anti-WNV-neutralizing antibodies target the lateral ridge of EDIII in distinct ways

To approximate the epitope recognized by anti-WNV antibodies, we performed competition binding experiments using surface plasmon resonance (SPR). By this method, neutralizing antibodies were classified into three main competition bins: W009, W010, W048 and W049 competed with one another, but not with W007 and W014, whereas W037 competed with all antibodies tested (Figures 4A, S4A, and S4B). To define the structural basis underlying these competition profiles and associated neutralization mechanisms, we solved high-resolution crystal structures of the antigen-binding fragment (Fab) from W010, W014, W037, and W049 in complex with the WNV_{EDIII} protein. Consistent with the SPR competition data, the antibodies adopted distinct binding orientations allowing recognition of distinct epitopes on EDIII (Figures 4B and 4C).

The structure of the W037 Fab-WNV_{EDIII} complex revealed recognition of the lateral ridge, encompassing the N-terminal region and the BC, DE, and FG loops of EDIII, which together span the EDI-EDIII interface⁵⁴ (Figures 4D–4G).

Similarly, W049 engaged the lateral ridge epitope in a manner closely resembling that of the mouse anti-WNV antibody E16, with primary contacts involving the A-strand and the BC and

hydrogen-bonding network on Thr332_{EDIII} and Asn368_{EDIII}. Additionally, the CDRL1 of W049 formed a stabilizing salt bridge between Glu30_{CDRL1} and Lys310_{EDIII}. Together, W037 and W049 represent canonical lateral ridge binding antibodies and share a binding orientation similar to potent monoclonal antibodies against several orthoflaviviruses (Figures S4C and S4D).

In contrast, W010 and W014 exhibited distinct binding poses not previously observed. The structure of the W010 Fab-WNV_{EDIII} complex revealed engagement of the A-strand, and of the BC and DE loops, mediated primarily through the CDRH3 and CDRL3 loops (Figure 5A). W010 buried a total surface area (BSA) of 705.5Å² on EDIII, with contributions from both heavy (360Å²) and light (345.6Å²) chains. Key CDRH3-interactions included a salt bridge between Glu100_{G-HC} and Lys310_{EDIII} on the A-strand, as well as side-chain and backbone interactions with residues in the BC and DE loops by Glu100_{E-HC} and Cys100_{B-HC}, respectively (Figures 5B and 5C). Lys310_{EDIII} was also engaged by CDRL3 residues Ser91_{LC} and Tyr92_{LC} (Figure 5D). Given that Lys310_{EDIII} accounts for ~14% of the total BSA of the epitope, low sequence conservancy at this position among the orthoflaviviruses of the JE serocomplex likely explains W010's lack of cross-reactivity (Figure 5E).

To gain insight about how W010 bound to and neutralized the virus, we used a previously determined cryo-electron microscopy (cryo-EM) structure of the WNV virion to model epitope recognition⁵⁵ (Figures S5A–S5C). In the context of the mature WNV virion, the W010 epitope was fully accessible, with W010 anchoring at the loop regions directly adjacent to the EDII domain of the same protomer that comprised the lateral ridge epitope. W010's pose provided a binding advantage whereby two Fab molecules could engage the WNV epitope at the 5-fold symmetry axis suggesting a mechanism for enhanced potency through avidity effects (Figure S5B). Consistent with this view, W010 IgG was superior to Fab in WNV neutralization (Figure S5D). Thus, both the mode of engagement and bivalent binding contribute to the potency of W010. Passaging the virus

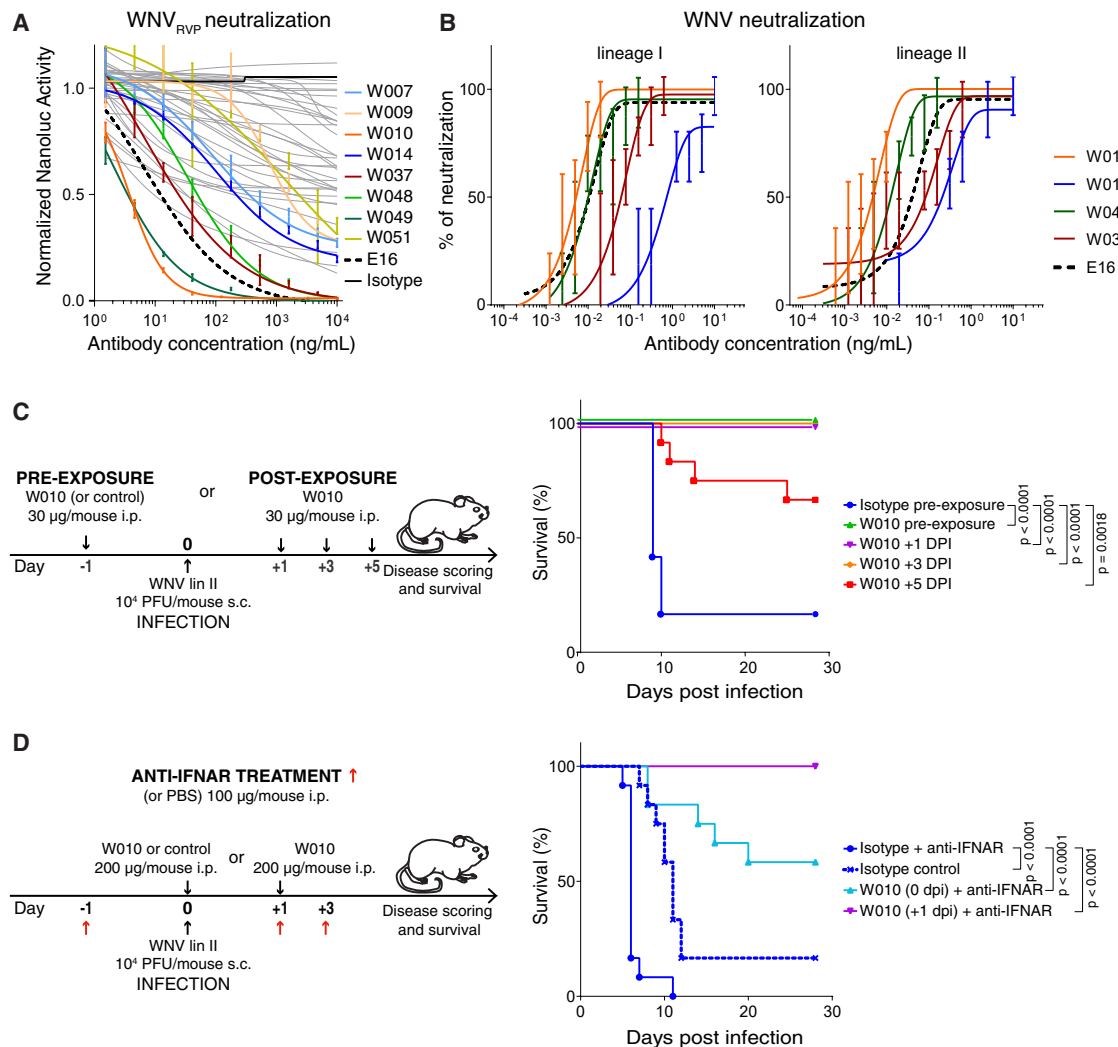


Figure 3. W010 potentially neutralizes WNV

(A) Neutralization of WNV_{RVP} by monoclonal antibodies. NanoLuc activity in the presence of serially diluted antibodies relative to no-antibody control is shown. Mean $\pm$ SD of triplicates. Representative of two experiments.

(B) Neutralization of virus isolates representing WNV lineages I and II. Representative neutralization curves by serially diluted antibodies. The percentage of neutralization was calculated based on the development of cytopathic effects 5 days after infection by comparing antibody-treated to non-treated control. Mean $\pm$ SD of octuplicates. Representative of two experiments. See also Figure S3A.

(C) Experimental timeline and survival curves for W010 pre- and post-exposure prophylaxis against lethal WNV lineage II infection of mice. Left, diagram of the experiment's timeline. Right, survival curves of each experimental group are compared. Two independent experiments with six mice per group in each experiment were combined and differences in survival were analyzed by log rank Mantel-Cox test. See also Figure S3B.

(D) Experimental timeline and survival curves for therapeutic W010 administration after lethal WNV lineage II infection of mice receiving anti-IFNAR antibodies. Left, diagram of the experiment's timeline. Right, mouse survival over time. Two independent experiments with six mice per group in each experiment were combined and differences in survival were analyzed by log rank Mantel-Cox test.

See also Figure S3C.

under subneutralizing concentrations of W010 *in vitro* led to the emergence of antibody-resistant variant carrying the amino acid change N368T, a residue contributing $\sim 11\%$ of the epitope's total BSA (Figure 5E; data not shown). While the structure revealed a direct hydrogen bond between N368 and the antibody, supporting its role in epitope recognition (Figure 5C), the structural data alone did not fully explain the magnitude of the ~ 100 -fold reduction in neutralization observed upon introduction of the N368T substitution (Figure S5E). However, the functional impor-

tance of this residue is supported by both the observed contact in the structure and the virus escape data.

W014 targets a protective epitope shared across the JE serocomplex

WNV_{EDIII} antibodies were next screened for the ability to cross-neutralize RVPs corresponding to related viruses of the JE serocomplex (see STAR Methods). W014 (clonally related to W007) was identified as a broad neutralizer of WNV (IC₅₀ value =

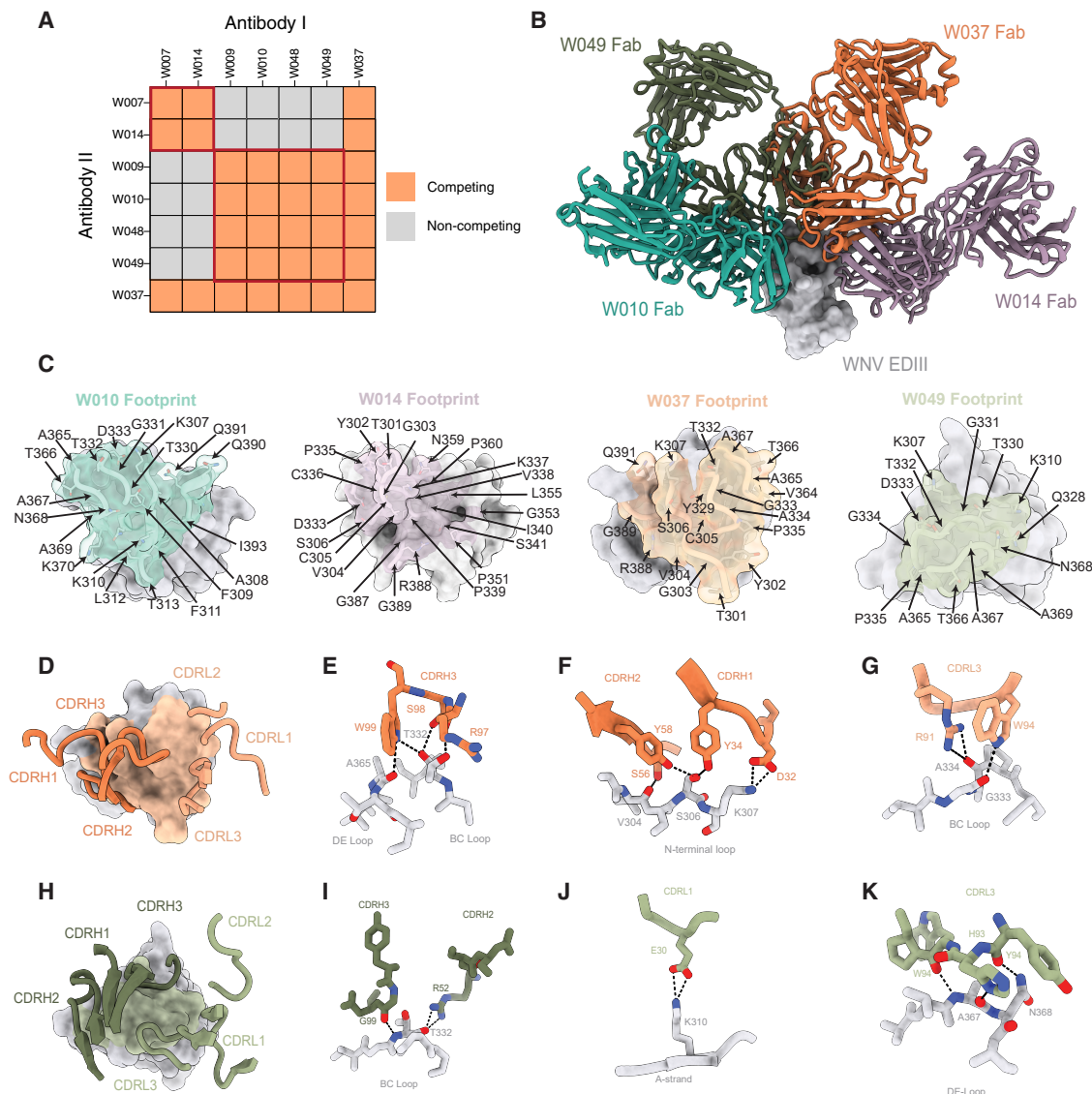


Figure 4. Neutralizing antibodies target distinct EDIII epitopes

(A) Epitope binning by competition SPR. Upon immobilization of antibody I, WNV_{EDIII} was bound, followed by antibody II. Summary of antibodies competing for the same epitope by this method. Related to [Figures S4A and S4B](#).

(B) Superposition of crystal structures of WNV_{EDIII} in complex with W010, W037, W014, and W049 Fabs resolved to 1.8, 2.1, 1.4, and 2.5 Å, respectively. Structures were aligned to the WNV_{EDIII}.

(C) The EDIII epitopes of W010, W014, W037, and W049 defined as interfacial residues in PDBePISA (see [STAR Methods](#)).

(D) W037 Fab CDR loops mapped onto the antibody footprint, which is highlighted in orange on the EDIII surface.

(E) Molecular interactions between W037 CDRH3 loop and the EDIII BC and DE loops. Trp99_{HC} mediates hydrogen bonding with backbone atoms Ala365_{EDIII} and Thr332_{EDIII}.

(F) W037 CDRH2 and CDRH1 residues form a series of potential hydrogen bonds with the N-terminal loop region of the lateral ridge epitope, including a common mode of engagement by the side chain of Tyr58_{HC} and Tyr34_{HC} with Ser306_{EDIII}.

(G) W037 CDRL3 engagement with the BC loop region of the lateral ridge epitope. Hydrophobic and hydrogen-bonding interactions are indicated.

(H) W049 Fab CDR loops mapped onto the antibody footprint, which is highlighted in green on the EDIII surface.

(I) W049 CDRH3 and CDRH2 contacts the BC loop. Hydrogen-bonding interactions between Gly99_{CDRH3} and Arg52_{CDRH2} with Thr332_{EDIII} in the BC loop are shown.

(J) W049 CDRL1 salt bridge formation between Glu30_{EDIII} and Lys310_{EDIII} in the A-strand of the lateral ridge.

(K) W049 CDRL3 contacts the DE loop. Charged electrostatic and hydrogen-bonding interactions involving DE loop residues Ala367_{EDIII} and Asn368_{EDIII}.

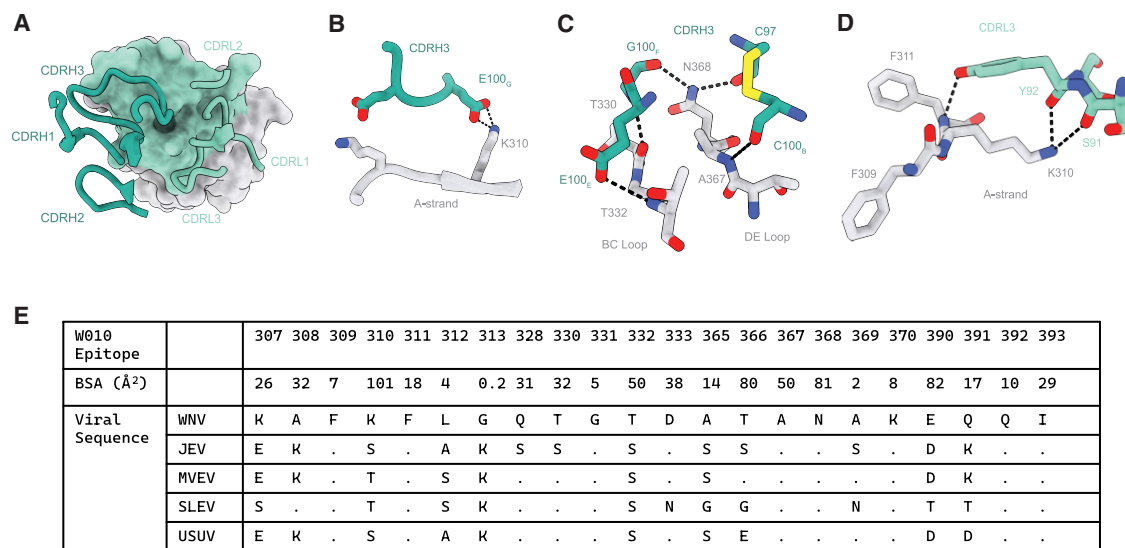


Figure 5. W010 targets a distinct neutralizing epitope on the EDIII

(A) W010 Fab CDR loops mapped onto the antibody footprint, which is highlighted in cyan on the EDIII surface.

(B) W010 CDRH3 interactions with WNV_{EDIII}. A salt bridge between residue Glu100_{G-HC} and Lys310_{EDIII} in the A-strand of the lateral ridge epitope is indicated.

(C) Hydrogen-bonding networks between W010 CDRH3 residues and the BC and DE loops of the EDIII lateral ridge epitope.

(D) W010 CDRL3 interactions with WNV_{EDIII}. Hydrogen bonds between Ser91-Tyr92_{CDRL3} and Lys310_{EDIII} are shown. Hydrogen bonds are represented as dashed lines between donor and acceptor atoms.

(E) Sequence contacts for the W010 epitope and associated combined V_H- and V_L- buried surface area. The WNV EDIII was used as the reference sequence for alignment with EDIII proteins of the JE serocomplex. Identical residues in the JE serocomplex are indicated with a period and differences are represented by the residue at that position.

102 ng/mL), JEV (IC₅₀ value = 43.6 ng/mL), MVEV (IC₅₀ value = 27.7 ng/mL), SLEV (IC₅₀ value = 4.3 ng/mL), and USUV (IC₅₀ value = 36.2 ng/mL), while W051 neutralized MVEV (IC₅₀ = 485 ng/mL) and SLEV (IC₅₀ = 12.8 ng/mL) (Figures 6A and S6A; Table S3). In line with these observations, sera from the three individuals, from whom antibodies were derived, also exhibited cross-neutralization of JEV, MVEV, SLEV, and USUV RVPs (Figure S6B).

Structural analyses of the W014 Fab-WNV EDIII complex revealed recognition of the N-terminal and FG loops, which are canonical components of the lateral ridge epitope, and the CC' and DD' loops associated with the CC' epitope (Figures 6B–6E). In contrast to W010, the W014 light chain made minimal contacts at the epitope, where interactions were mediated by CDRH1, CDRH2, and CDRH3 loops (V_H: 566 Å², V_L: 27.9 Å²). W014 inserted an aromatic motif at the tip of the CDRH3 (Trp100_{A-HC}-Tyr100_{C-HC}) into a cleft framed by Lys337_{EDIII}, Pro339_{EDIII}, and Pro351_{EDIII}, which are highly conserved residues in the JE serocomplex (Figures 6C and 6F). Additionally, W014 formed backbone interactions at the lateral ridge whereby CDRH1 residues Arg28_{HC} and Tyr32_{HC} hydrogen bond with the carbonyls of Val304_{EDIII} and Thr301_{EDIII}, respectively, and CDRH2 residue Tyr52_{HC} inserted into a pocket formed by the FG loop and N-terminal loop (Figures 6D and 6E). Collectively, engagement with strictly conserved EDIII residues and extensive backbone interactions in the W014 epitope likely facilitated cross-reactive binding among the orthoflaviviruses of the JE serocomplex (Figure 6F). However, this cryptic epitope was buried in the context of the mature virions, likely explaining W014's

weaker neutralization potency relative to W010 (Figure S5C). Thus, in addition to potent but monospecific antibodies such as W010, infection by WNV can induce antibodies that are broadly effective against other members of the JE serocomplex.

In agreement with the RVP neutralization data, W014 neutralized authentic WNV lineages I and II (IC₅₀ values of 773 and 143 ng/mL, respectively, as determined by cytopathic effect assays; Figure 3B), as well as WNV, JEV, MVEV, and SLEV by an immunofluorescence-based assay, albeit requiring higher antibody concentrations (Figures S3A and S6C). Finally, to assess the efficacy of W014 *in vivo*, we infected mice with WNV 1 day after antibody administration. W014 significantly prevented mice from dying (*p* = 0.002; Figures 6G and S6D), demonstrating that the conserved epitope targeted by W014 mediates protection in a preclinical model.

DISCUSSION

Infection with mosquito-borne WNV can result in severe neurological complications (i.e., encephalitis, meningitis, and flaccid paralysis) with potentially fatal outcomes. In the absence of specific treatments, WNV remains a public health concern, especially due to its continuous geographic expansion.^{21,56–58} Herein, we described the identification of potent human neutralizing antibodies with therapeutic potential for prevention or treatment of WNV-associated disease. Some of these antibodies also exhibited cross-neutralizing activity against related orthoflaviviruses in the JE serocomplex, including JEV,

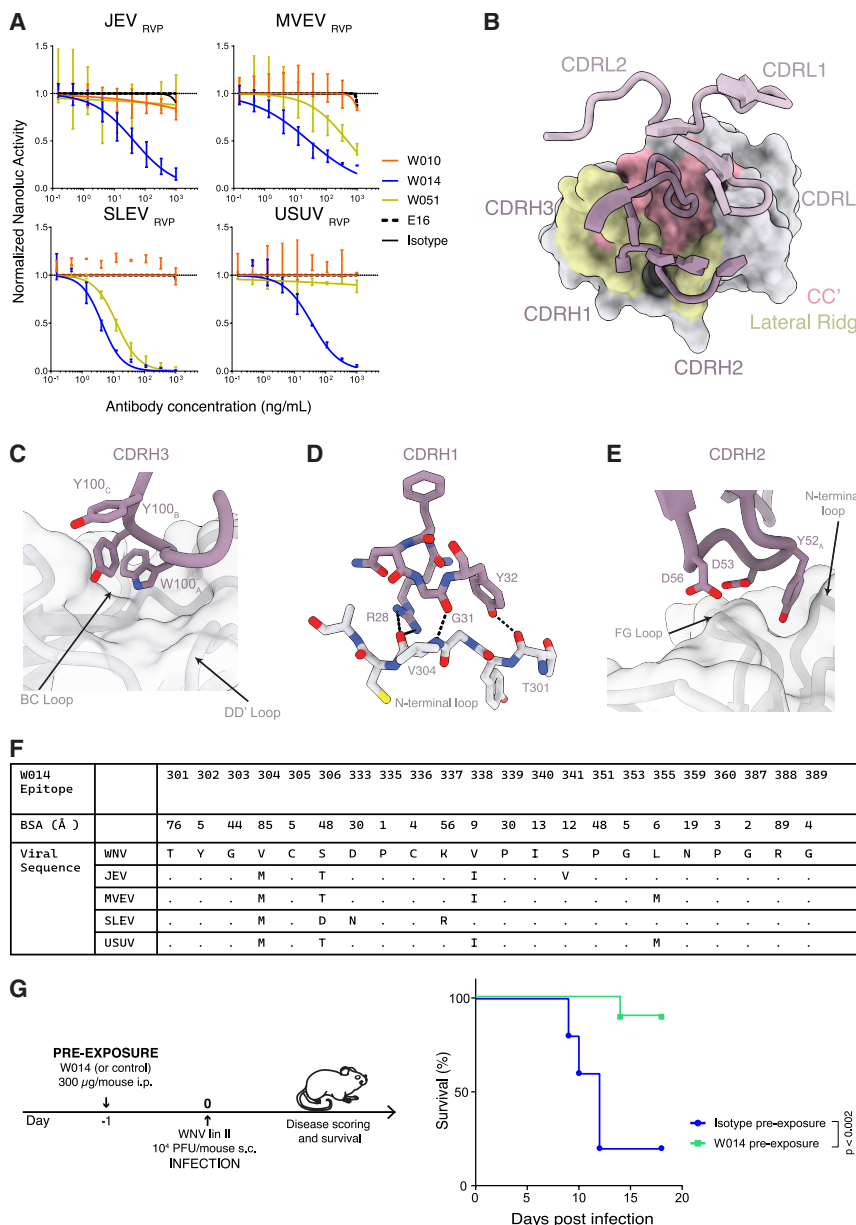


Figure 6. W014 targets a protective epitope shared across the JE serocomplex

(A) Neutralization of RVPs corresponding to WNV-related orthoflaviviruses JEV, MVEV, SLEV, and USUV. NanoLuc activity in the presence of serially diluted antibodies relative to no-antibody control is shown. Mean $\pm$ SD of triplicates. Representative of two experiments.

(B) Interface between W014 CDR loops and WNV EDIII lateral ridge (yellow) and CC' (pink) epitopes is shown.

(C) Surface and cartoon representation of the CC' hydrophobic pocket recognized by the aromatic residues at the tip of the W014 CDRH3 loop.

(D) Interaction between W014 CDRH1 and the N-terminal loop is shown. Hydrogen-bonding contacts are depicted with dashed black lines.

(E) W014 CDRH2 buries Tyr52_A into a hydrophobic cleft formed by the N-terminal and the FG loops of the lateral ridge epitope.

(F) Sequence contacts for W014 epitope and associated combined V_H- and V_L- buried surface areas are shown. The WNV EDIII is used as the reference sequence for alignment with EDIII proteins of the JE serocomplex. Identical residues in the JE serocomplex are indicated with a period and differences are represented by the residue at that position.

(G) Efficacy of W014 in pre-exposure prophylaxis against lethal WNV lineage II infection is shown. Left, diagram of the experiment's timeline. Right, survival curves of each experimental group are compared. One experiment with ten (W014) and five (isotype) mice per group. Log rank Mantel-Cox test.

MVEV, SLEV, and USUV, all of which are a medically relevant source of severe human diseases. Moreover, we reported that the presence of autoantibodies neutralizing IFN-Is did not impair the development of antiviral antibody responses.

Recent studies have associated the presence of autoantibodies neutralizing IFN-Is, particularly IFN- α 2 and/or - ω , with increased risk of severe disease and fatal outcome following orthoflavivirus infection, as well as adverse events following vaccination against yellow fever.^{13–15} In cases of WNV encephalitis, around 40% of poor clinical outcomes were linked to IFN- α 2 and/or IFN- ω neutralizing autoantibodies,⁸ a frequency comparable to that observed in the cohort reported herein (18%). These findings underscore the key role of IFN-Is in antiviral defenses^{59–61} and are consistent with observations that mice genetically defective in IFN signaling,^{16,62} such as *IFN-*

$\alpha/\beta R^{-/-}$,⁶³ *Stat1^{-/-}*,¹⁶ and *IRF-7^{-/-}*,⁶⁴ are more susceptible to WNV infection. We found that antiviral antibodies were not impaired by the presence of IFN- α 2 and/or IFN- ω neutralizing autoantibodies, which is consistent with reports that IFN- α 2 and/or IFN- ω autoantibodies do not interfere with coronavirus disease 2019 (COVID-19) vaccinal responses.^{65–67} In fact, individuals with

IFN- α 2 and/or IFN- ω neutralizing autoantibodies in our study exhibited significantly higher WNV EDIII-specific antibodies, potentially reflecting higher viremia due to functional impairment of IFN- α 2 and/or IFN- ω signaling that drives a more robust humoral response. This hypothesis could be tested in future studies by comparing viral and antigenic loads in IFN- α 2 and/or IFN- ω competent patients versus those that are functionally impaired. However, more antiviral antibodies did not translate into improved virus neutralizing activity by the serum.

Human antibodies specific to the EDIII of other orthoflaviviruses were previously shown to possess strong virus neutralizing capabilities and potential for clinical development. For example, T025, a TBEV_{EDIII} antibody, is protective even at low doses in murine models of tick-borne encephalitis,³⁴ while ZIKV_{EDIII} monoclonal antibodies Z004 and Z021 show efficacy

in mouse as well as in macaque models of Zika congenital syndrome.^{32,68} These anti-EDIII antibodies, whose efficacy is restricted to a single or only few orthoflaviviruses, share some structural features including the usage of the lateral ridge epitope at the hinge of the EDI–EDIII regions like observed for W037 (Figure S4D).

W010 and W049, the two most potent WNV-neutralizing antibodies from this study, shared key epitope contact features, including salt bridge formation with Lys310_{EDIII} and hydrogen-bonding networks involving Thr332_{EDIII} and Asn368_{EDIII}. Despite these similar contact points, W010 used a divergent binding pose to engage components of the lateral ridge epitope, which may have contributed to its enhanced neutralization potency. To further explore this possibility, we compared W010 with the WNV_{EDIII} antibody of mouse origin (E16) under clinical development, which recognizes the canonical lateral ridge epitope but has decreased neutralization potency^{41,69} (Figures 3B and S4D). In orthoflaviviruses, effective neutralization typically requires engagement of approximately 30 antibody molecules targeting the lateral ridge of EDIII based on the virion's size and icosahedral symmetry.⁷⁰ Both E16 and W010 bind this accessible epitope and can exceed the neutralization threshold, with E16 reported to engage up to 120 binding sites.⁷⁰ Our new data indicated that both antibodies are capable of bivalent binding, suggesting that differences in avidity alone do not fully account for the increased neutralization potency of W010 (Figure S5D). Instead, we speculate that the distinct binding orientation of W010 may allow more favorable engagement of EDIII across both mature and immature virions, potentially increasing epitope accessibility and functional occupancy at critical sites such as the 5-fold symmetry axis. Together, these features are likely contributors to the enhanced neutralization potency observed for W010 relative to E16.

In contrast to EDIII antibodies that were potent but monospecific, some EDIII antibodies were also discovered that possessed broader spectrum efficacy. W014, for example, neutralized RVPs (and to a lower efficacy authentic viruses) corresponding to nearly all orthoflaviviruses that are transmitted by *Culex* spp. mosquitoes and relevant for human disease (WNV, JEV, MVEV, SLEV, and USUV). Similar broadly acting antibodies have been identified that are effective against the tick-transmitted pathogenic orthoflaviviruses TBEV, OHFV, and KFDV (T056 and T058)³⁴ or those transmitted by *Aedes* spp. mosquitoes ZIKV and the four serotypes of DENV (Z039³²; unpublished data). Structural analyses aimed at elucidating the molecular basis of cross-reactivity by broadly neutralizing anti-EDIII antibodies revealed that these antibodies recognize the lateral ridge and CC' epitopes. Notably, W014 adopted a binding orientation that enabled interaction with highly conserved residues within the CC' epitope, which is shared across members of the JE serocomplex.⁷¹ However, this conserved epitope is not exposed on either mature or immature virions, suggesting that conformational rearrangements of the virus envelope are required to transiently expose the CC' site. A similar binding mode was previously observed for the anti-DENV antibody E111, which supports the hypothesis that CC'-targeting antibodies neutralize infection by recognizing conformational intermediates that arise as the E protein transitions from homodimeric to homotrimeric states during

membrane fusion.⁷² Collectively, the antibodies described here reveal two principal epitope-targeting strategies that can elicit potent and, in some cases, broadly neutralizing responses. These findings also provide a structural and mechanistic framework to inform the design of broad-spectrum “prototype” immunotherapies and vaccines, with the potential to protect against both currently circulating orthoflaviviruses and those that may emerge in the future that pose a threat to human health.

The *in vivo* experiments highlighted the distinctive efficacy of W010, even when administered prophylactically at low dose (30 µg/mouse) or therapeutically several days after lethal WNV challenge of mice. A higher dose (200 µg/mouse) was also protective in mice with impaired IFN signaling. In humans, WNV viremia is most often accompanied by mild symptoms, which are reported to begin after an incubation period of 2–14 days.^{5,73,74} It is conceivable that administration of W010 at this time could reduce viral loads, thus limiting the risk of progression to the neurologic phase of the disease. It is also possible that prophylactic administration of antibodies in the context of WNF/WND seasonal outbreaks or in response to early warning^{75,76} would benefit especially at-risk populations, such as older adults, immunocompromised individuals, and those with functionally impaired type I IFN signaling. Similarly, W014 protected mice against WNV and future studies will assess whether its administration could lessen the risk of disease by JEV and other orthoflaviviruses of the JE serocomplex.^{77–79}

In summary, the identification of human antibodies that are potent and broadly active against WNV and related orthoflaviviruses provides relevant knowledge to the development of medical countermeasures against severe diseases that lack specific treatments and represent an important unmet medical need globally.

Limitations of the study

It was not possible to determine the CCR5 genotype of the individuals in the West Nile disease cohort (polymorphisms at CCR5 are a risk factor for severe disease), and thus the relationship between CCR5 alleles and serologic features, including the presence of type I IFN-neutralizing autoantibodies, could not be evaluated. Although an escape variant of WNV from W010 treatment was identified in tissue culture experiments, it remains to be determined whether escape from W010 can also occur *in vivo*.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Davide F. Robbiani (drobbiani@irb.usi.ch).

Materials availability

Expression plasmids and other materials generated in this study will be shared upon request and as allowed by applicable laws; distribution may require a material transfer agreement.

Data and code availability

All data reported in this paper, including antibodies sequences, are available in the main text or the [supplemental information](#) or will be shared by the [lead contact](#) upon request. The atomic models and corresponding structure factor files for all structures are available in the Protein Data Bank (PDB) under the following accession codes: W010 Fab-WNV EDIII (PDB: 9ZRM), W014

Fab-WNV EDIII (PDB: 9ZRN), W037 Fab-WNV EDIII (PDB: 9ZRO), and W049 Fab-WNV EDIII (PDB: 9ZRP). The data are publicly available from the date of publication. This paper does not report any original code. Any additional information required to reanalyze the data reported in this work is available from the [lead contact](#) upon request.

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AUTHOR CONTRIBUTIONS

D.F.R. and D.R. conceptualized the study. T.C.R., T.F., Z.I.C., K.G., B.C., F.B., L.S., S.T., V.H., S.B., L.V., M.P., B.G.H., P.B., D.R., C.O.B., and D.F.R. designed and analyzed the experiments. P.B., J. Grujić, D.M., M.V., S.S., and D.L. designed human subject research protocols. T.C.R., T.F., Z.I.C., J.C., K.G., B.C., F.B., L.S., D.J., S.T., R.K., A.R.E.R., M.C., E.G.N., J.M., and Z.K. produced reagents and carried out experiments. M.R.M. provided unpublished reagents. T.C.R., R.T., and T.K. provided reagents. Z.B.-O., J. Grujić, M.V., and Z.L. collected and processed blood samples. D.M., J. Grujić, Z.B.,

M.V., Z.L., D.L., S.S., M.B., L.P.-D., B.P.J., and J. Gavrilović recruited participants and executed human subject research protocols. T.C.R., J.C., J. Grujić, D.M., Z.B.-O., M.V., Z.L., D.L., S.S., M.B., L.P.-D., B.P.J., and J. Gavrilović processed human samples. Z.I.C. and A.R.E.R. performed crystallography experiments. Z.I.C. and C.O.B. performed structural analysis. T.F., M.C., J.H., J.S., V.H., and D.R. performed *in vivo* experiments. S.G.M. performed bioinformatic analysis. T.C.R., T.F., Z.I.C., J.C., D.R., C.O.B., and D.F.R. wrote the manuscript with input from all coauthors.

DECLARATION OF INTERESTS

The Institute for Research in Biomedicine has filed a provisional patent application in connection with this work.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, the authors used ChatGPT by OpenAI to improve language and readability of portions of the manuscript. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-IgG (human) sheep polyclonal antibody HRP	VWR	Cat#NA933
Human recombinant antibody E16	Oliphant et al. ⁴¹	N/A
Human recombinant antibody Z021	Robianni et al. ³²	N/A
Human recombinant antibodies hr2.016, hr2.024, sd1.040	Bianchini et al. ⁸⁰	N/A
Human recombinant antibody 10-1074	Mouquet et al. ⁸¹	N/A
Human recombinant antibody ED38	Meffre et al. ⁸²	N/A
Human recombinant antibody P003	Cervantes Rincón et al. ⁵²	N/A
Biotin-SP anti-IgG (human) goat polyclonal antibody	Jackson Immunology	Cat#109-067-008; RRID:AB_2632441
anti-CD14-APC-eFluor 780	Thermo Fisher Scientific	Cat#47-0149-42; RRID:AB_1834358
anti-CD16-APC-eFluor 780	Thermo Fisher Scientific	Cat#47-0168-41; RRID:AB_11219083
anti-CD8-APC-eFluor 780	Invitrogen	Cat#47-0086-41; RRID:AB_2573944
anti-CD3-APC-eFluor 780	Thermo Fisher Scientific	Cat#47-0037-42; RRID:AB_2573936
anti-CD20-PE-Cy7	BD Biosciences	Cat#335828; RRID:AB_2868689
Mouse anti-Flavivirus group antigen antibody, clone D1-4G2-4-15 (4G2)	Sigma-Aldrich	Cat#MAB10216-I
Goat anti-mouse antibody	Invitrogen	Cat#A32723
Anti-mouse IFNAR-1	BioXCell	Cat#BP0241; RRID:AB_2894815
Bacterial and virus strains		
WNV Eg-101	Collection of Arboviruses, CAS, CZ	N/A
WNV 13-104	Provided by Zdeněk Hubálek and Ivo Rudolf	N/A
UVE/MVEV/UNK/AU/3329	EVAg	Cat#001v-EVA145
UVE/SLEV/UNK/US/MSI-7	EVAg	Cat#001v-EVA128
UVE/JEV/2009/LA/CNS769	EVAg	Cat#001V-02217
<i>E. coli</i> Rosetta 2 (DE3)	Sigma-Aldrich	Cat#71397
Biological samples		
Sera from WND individuals	This paper	N/A
Sera from blood donors from Serbia	This paper	N/A
Sera from blood donors from Switzerland	This paper	N/A
Chemicals, peptides, and recombinant proteins		
Medium 199	Gibco	Cat#11150059
Expi293 medium	Gibco	Cat#A1435101
Soy hydrolysate	Sigma-Aldrich	Cat#58903C
TMB	Thermo Fisher Scientific	Cat#34021
Lipopolysaccharides from <i>Escherichia coli</i>	Sigma-Aldrich	Cat#L2637
Insulin solution human	Sigma-Aldrich	Cat#I9278
Deoxyribonucleic acid—cellulose double-stranded from calf thymus DNA	Sigma-Aldrich	Cat#D8515

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Hemocyanin from <i>Megathura crenulata</i> (keyhole limpet)	Sigma-Aldrich	Cat#H8283
Human IFN- α 2	This paper	N/A
IFN- α 2	Novus Biologicals	Cat#NBP2-34971
IFN- ω	Novus Biologicals	Cat#NBP2-35893
SuperScript III Reverse Transcriptase	Invitrogen	Cat#18080-044
Streptavidin-PE conjugate	eBioscience	Cat#12-4317-87
Streptavidin-Alexa Fluor 647 conjugate	BioLegend	Cat#405237
Biotinylated Ovalbumin	Sigma-Aldrich	Cat#A5503-1G
Streptavidin-BV711	BD Horizon	Cat#563262
TBEV(WE)-, TBEV(FE)-, TBEV(Si)-, POWV(LB)-, POWV(DTV)-, OHFV-, KFDV-, LIV- and LGTV-EDIII proteins	Agudelo et al. ³⁴	N/A
ZIKV-, DENV1, DENV2, DENV3, DENV4, YFV(17D)-, WNV(NY99)-EDIII proteins	Robbiani et al. ³²	N/A
USUV-, JEV-, SLEV-, MVEV-EDIII proteins	Cervantes Rincón et al. ⁵²	N/A
WNV(lineage II)-, JEV (p3)- EDIII proteins	This paper	N/A

Critical commercial assays

Superdex-75 16/600 column	Cytiva	Cat#28989333
QuikChange Multi Site-Directed Mutagenesis Kit	Agilent Technologies	Cat#200514
BirA Biotin-Protein Ligase kit	Avidity	Cat#BirA500
Ni-NTA affinity column	Thermo Fisher Scientific	Cat#88222
Superdex S200 16/60 column	Cytiva	Cat#28989335
Renilla luciferase substrate (EnduRen)	Promega	Cat#E6481
Pan B-cell isolation kit	Miltenyi Biotec	Cat#130-101-638
LS magnetic columns	Miltenyi Biotec	Cat#130-042-401
ThermalShift Assay kit	Thermo Fisher Scientific	Cat#4461146
Nano-Glo Luciferase Assay System	Promega	Cat#N1120
QIAamp Viral RNA Mini Kit	QIAGEN	Cat#52904
Advanced Kit for West Nile Virus	Genesig	Cat#Z-Path-WNV-EASY
OneStep qRT-PCR Master Mix	Oasig	Cat#Z-oasig-onestep-150
Superdex 200 10/300 column	Cytiva	Cat#28990944
96-well crystallization screens	Hampton Research	Cat#HR2-086, HR2-139 and HR2-130

Deposited data

W010-WNV EDIII structure	This paper	PDB: 9ZRM
W014-WNV EDIII structure	This paper	PDB: 9ZRN
W037-WNV EDIII structure	This paper	PDB: 9ZRO
W049-WNV EDIII structure	This paper	PDB: 9ZRP

Experimental models: Cell lines

Expi293F	Thermo Fisher Scientific	Cat#A14527
HEK293T	ATCC	Cat#CRL-1573
Huh-7.5	Blight et al. ⁸³	N/A
A549	ATCC	Cat#CRM-185
A549-based AIR	Groen et al. ⁴⁹	N/A

Experimental models: Organisms/strains

BALB/cOlaHsd	Envigo	RRID:IMSR_ENV:HSD-162
Fc γ R/FcRn humanized mice	Borghi et al. ⁵³	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligonucleotides		
Primers for antibody cloning by the SLIC method	Robbiani et al. ³²	N/A
Primers for NanoLuc amplification forward 5'-GTACGCGTATGGTCTTCACACTCGAAGA-3'	This paper	N/A
Primers for NanoLuc amplification reverse 5'-GAAGTTACGCGCCGTCGCCAGAATGCGTTCGCACA-3'	This paper	N/A
Primers to sequence the virus E gene WNV; WNV_1A CCTGGTGGCACCAGCATAACAGC + WNV_1B GTGGTGCTCCAGCACTGCTCC	This paper	N/A
Primers to sequence the virus E gene WNV; WNV_2A GCGAAGTCCTTCCTGGTTCACCGA + WNV_2B CACAGCCTGTGTCAGCATGGACG	This paper	N/A
Recombinant DNA		
IG γ 1-, IG κ or IG λ -expression vectors	Tiller et al. ⁸⁴	N/A
pWNV/TX02/CprME	Laboratory of T. Pierson - NIH	N/A
pWNVII-Rep-REN-IB	Laboratory of T. Pierson - NIH	N/A
pWNVII-Rep-NanoLuc	This paper	N/A
pJEV-SA-14-CprME	This paper	N/A
pMVEV-MK6684-CprME	This paper	N/A
pSLEV-Parton-CprME	This paper	N/A
pUSUV-Vienna2001-CprME	This paper	N/A
Software and algorithms		
GraphPad Prism (v.10)	GraphPad Software	RRID:SCR_002798
FlowJo (v.10.10.0)	BD Biosciences, FlowJo	RRID:SCR_008520
IgBlast	National Center for Biotechnology Information (NCBI), National Institutes of Health	RRID:SCR_002873
Biacore Insight Evaluation Software (v.5.0.18.22102)	Cytiva (Biacore)	N/A
CellReporterXpress	Molecular Devices	RRID:SCR_025681
XDS	XDS Program, Package; Kabsch ⁸⁵	RRID:SCR_015652
CCP4 - AIMLESS	CCP4 Program Suite; Evans and Murshudov ⁸⁶	RRID:SCR_015747
Phenix	Phenix Project; Adams et al. ⁸⁷	RRID: SCR_014224
Coot	University of York; Emsley and Cowtan ⁸⁸	RRID: SCR_014222
IMGT/V-QUEST	IMGT, the international ImMunoGeneTics information system	RRID: SCR_010749
ChimeraX	University of California, San Francisco; Pettersen et al. ⁸⁹	RRID: SCR_015872
PDBePISA	European Molecular Biology Laboratory – European Bioinformatics Institute (EMBL-EBI)	RRID:SCR_015749
Biorender	Biorender	https://BioRender.com
Other		
EPOCH2 microplate reader	BioTek	N/A
SpectraMax Plus spectrophotometer	Molecular Devices	N/A
QuantiStudio 6 flex thermal cycler	Thermo Fisher Scientific	N/A
PerkinElmer EnVision plate reader	PerkinElmer	N/A
FACSymphony S6 cell sorter	BD Biosciences	N/A
Biacore 8K	Cytiva	N/A
GloMAX Discover System reader	Promega	N/A
ImageXpress Pico	Molecular Devices	N/A

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Ethical statement

The study was approved by the ethical committees of University Clinical Centre of Vojvodina (approval No. 00–59), Blood Transfusion Institute of Vojvodina (approval No. 02-01/23), University Clinical Centre of Kragujevac (approval No. 01/22-389), and Pasteur Institute Novi Sad (approval No. 10-49/1). The research was conducted in compliance with the principles outlined in the Declaration of Helsinki and adhered to the Patient Rights Law of the Republic of Serbia.

Study participants

Eligible patients presented at the Clinics for Infectious Diseases of Niš, Novi Sad or Kragujevac during 2022 and 2023 with signs, symptoms and laboratory findings consistent with WNF and/or WND. All cases of WNF/WND were considered probable if the patient met any of the following clinical criteria: (i) clinical presentation of encephalitis and/or meningitis and/or fever $\geq 38^{\circ}\text{C}$ with (ii) presence of anti-WNV IgG in serum with seroconversion or a 4-fold increase in IgG titre on 2 subsequent samples (Euroimmun, Lubeck, Germany; EI 2662-9601 G). Demographic and clinical information on the cohort can be found in [Table S1](#). Healthy, orthoflavivirus naïve samples were obtained in Switzerland (Comitato Etico del Canton Ticino: CE-3428). All study participants provided informed consent.

Mice

Six-week-old female BALB/cOlaHsd mice were purchased from Envigo and housed in a BSL-3 facility under standard conditions (six mice per cage) with ad libitum access to food and water. Fc γ R/FcRn humanized mice were generated and characterized previously,⁵³ and maintained at the Comparative Bioscience Center at the Rockefeller University at a controlled ambient temperature environment with 12-h dark/light cycle.

Cell lines

All mammalian cell lines were grown at 37°C and supplemented with a mixture of antibiotics (100 U/mL penicillin and 100 $\mu\text{g/mL}$ streptomycin). HEK293T cells, a female human embryonic kidney-derived cell line, were cultured in Dulbecco's Modified Eagle Medium (DMEM) 10% FBS at 5% CO_2 . Expi293F cells, a female HEK293-derived suspension cell line, were cultured in Expi medium (Gibco, A1435101) at 8% CO_2 . Huh-7.5 cells,⁸³ a male human hepatocyte-derived cell line were cultured in DMEM supplemented with 10% FBS at 5% CO_2 . A549 cells (ATCC CRM-185), a male human lung epithelial carcinoma cell line, and A549-based AIR cells⁴⁹ were cultured in DMEM 10% FBS at 5% CO_2 . The sex of the cell lines was assigned based on supplier information and/or published cell-line provenance, as indicated in the [key resources table](#). Cell lines were not independently authenticated in our laboratory; their identity was based on supplier documentation and published provenance.

Viruses

Eg-101, belonging to WNV lineage I, was originally isolated from human serum in Egypt in 1951,⁹⁰ and was provided by the Collection of Arboviruses, Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, České Budějovice, Czech Republic. WNV strain 13-104, a representative of lineage II, was isolated from a *Culex modestus* mosquito in the Czech Republic in 2013⁹¹ and was kindly provided by Professors Zdeněk Hubálek and Ivo Rudolf, Institute of Vertebrate Biology, Czech Academy of Sciences, Valtice, Czech Republic. MVEV strain UVE/MVEV/UNK/AU/3329 (EVAg accession no. 001v-EVA145), SLEV strain UVE/SLEV/UNK/US/MSI-7 (EVAg accession no. 001v-EVA128), and JEV strain UVE/JEV/2009/LA/CNS769 (EVAg accession no. 001V-02217) were obtained from the EVAg and propagated in C6/36 prior to use.

METHOD DETAILS

Blood sample collection and serum extraction

After informed consent was obtained, 3 mL of blood was collected in BD Vacutainer SST Tubes (BD, Franklin Lakes, NJ, United States). Blood samples were allowed to clot at room temperature and, after centrifugation at $2000 \times g$ for 10 min, serum was extracted and stored at -80°C until further use. Prior to use, samples were heat-inactivated (56°C for 1h) and then stored at 4°C thereafter. Orthoflavivirus naïve serum samples were obtained from healthy individuals with no history of orthoflavivirus infection or vaccination, and no IgG seroreactivity to WNV_{EDIII} protein in preliminary ELISA.

Peripheral blood mononuclear cells (PBMCs) donor selection, large blood sampling and cell purification

Study participants were contacted during convalescence, starting from those with the highest serum neutralizing activity by the WNV_{RVP} assay, and invited to provide a large blood sample. Exclusion criteria for this part of the study were the same as those used for routine blood donors. Briefly, individuals with systemic diseases including diabetes and cardiovascular diseases, those aged below 18, or those with hemoglobin concentration $<125\text{ g/L}$ (female) and $<135\text{ g/L}$ (male) were excluded from the study. A detailed list of exclusion criteria (i.e., contraindications for blood donation) can be found in the Official Gazette of the Republic of Serbia.⁹² After determining hemoglobin concentration (using the HemoCue Hb 301 System, HemoCue AB, Angelholm, Sweden), the candidates completed a blood donation questionnaire, were interviewed by a physician and underwent physical examination

to establish suitability for large blood sampling. If successful up to this point of the selection, the candidate's blood was additionally tested for (i) complete blood count (Celltac Alpha MEK-6500K automated hematology analyzer, Nihon Kohden, Tokyo, Japan); (ii) coagulation status (i.e., prothrombin time, international normalized ratio, and activated partial thromboplastin time; on a Siemens BCS XP fully automated hemostasis analyzer, Siemens Healthcare GmbH, Erlangen, Germany); (iii) concentration of total proteins, albumins, immunoglobulins (isotypes G, M, and A), alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl transferase, total and direct bilirubin, urea, creatinine, and C-reactive protein using spectrophotometry on a Cobas Integra 400 plus fully automated analyzer (Roche Diagnostics International AG, Rotkreuz, Switzerland). In case of satisfactory findings (i.e., results within reference values), the consenting convalescent study participant was enrolled as PBMC donor and 350 mL of blood collected by venipuncture (Double Blood Bag CPDA 350 mL, Terumo Penpol Pvt Limited, India). The large blood sampling occurred approximately 10 months after hospital discharge (range: 9–11 months). Extraction of PBMCs was initiated within 5 hours after blood donation and was performed by gradient centrifugation using Histopaque. PBMCs were aliquoted in 90% Fetal Bovine Serum (FBS) and 10% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen as previously described,⁸⁰ until further processing.

Envelope domain III (EDIII) protein expression, purification and biotinylation

EDIII antigens were expressed in *E. coli* Rosetta 2 cells (DE3), purified from inclusion bodies, and refolded as previously detailed.³² Briefly, protein expression was induced with 1 mM isopropyl β -D-1-thiogalactopyranoside at 30°C for 4 hours. Cells were lysed, and the insoluble fraction containing inclusion bodies solubilized and refolded in a buffer containing 400 mM L-arginine, 100 mM Tris-base (pH 8.0), 2 mM ethylenediaminetetraacetic acid (EDTA), 0.2 mM phenylmethylsulfonyl fluoride, 5 mM reduced glutathione, 0.5 mM oxidized glutathione at 4°C. Refolded proteins were purified via size exclusion chromatography using a Superdex 75 column (Cytiva) in PBS buffer. The EDIII proteins were finally concentrated to 10–20 mg/mL. Codon-optimized EDIII expression plasmids corresponding to Orthoflaviviruses transmitted by *Culex* spp. mosquitoes (WNV lineage I, JEV, MVEV, SLEV and USUV), by ticks (POWV, KFDV, LGTV, LIV and OHFV) as well as by *Aedes* spp. mosquitoes (DENV serotypes 1–4, ZIKV, YFV) were previously reported.^{32,34,52} The plasmid to express the EDIII of WNV lineage II (residues 590–690 of GenBank: UTQ11699.1) was obtained by site-directed mutagenesis of the plasmid encoding for the EDIII of WNV lineage I using the QuikChange Multi Site-Directed Mutagenesis Kit (Agilent Technologies) following manufacturer instructions. For the purification of antigen-specific memory B cells, the WNV lineage I EDIII was modified to include an Avi-tag following the C-terminal 6XHis-tag. The Avi-tag enabled site-directed biotinylation using the biotin-protein ligase standard reaction kit (BirA500), according to the manufacturer's instructions (Avidity).

Recombinant human IFN- α 2 production and purification

An expression plasmid encoding human IFN- α 2 was generated by cloning the coding sequence of human IFN- α 2, fused at the C-terminus to an 8 $\times$ His tag and AviTag into the pcDNA3.1 mammalian expression vector. Plasmid transfection was performed using linear polyethylenimine hydrochloride (PEI-MAX; MW 40,000; Polysciences, 24765). For 300 mL Expi293F cell culture, 300 μ g of plasmid DNA was mixed with 3 mg of PEI-MAX in 30 mL of OPTI-MEM medium (Gibco, 51985026) and incubated for 20 minutes at room temperature before being added dropwise to the cell suspension. On day 2 post-transfection, 100 mL of Expi293 expression medium (Gibco, A1435101) supplemented with 2 mL of soy hydrolysate (Sigma-Aldrich, 58903C) and 0.67 mL of D-Glucose (Sigma-Aldrich, G8769) was added. On day 4 post-transfection, cells were pelleted by centrifugation at 2500 $\times$ g for 30 min at 4°C, and the clarified supernatant was collected. It was then adjusted with 50 mM Tris-HCl (pH 7.5), 5 mM NiSO₄, and 350 mM NaCl, filtered through 0.2 μ m membranes, and loaded onto a 2 mL Ni-NTA affinity column (Thermo Fisher Scientific, 88222) pre-equilibrated with 10 mL equilibration buffer (50 mM Tris-HCl, pH 7.5, 350 mM NaCl). The resin was then washed with 50 mL wash buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 40 mM Imidazole) and bound protein was eluted with elution buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 250 mM Imidazole) in 10–15 fractions of 1 mL each. Fractions with the highest protein concentration, estimated by Bradford assay, were pooled and concentrated to 2 mL using a Vivaspin 20 centrifugal concentrator (10 kDa MWCO; Sigma-Aldrich, Z614653). The concentrated eluate was filtered (0.2 μ m) and further purified by size-exclusion chromatography using a Superdex S200 16/60 column (Cytiva) pre-equilibrated with 1 $\times$ PBS. After quality control analysis, the purified protein was aliquoted and stored at -80°C until use.

ELISA

Detection of serum IgG or monoclonal antibody binding to EDIII antigen was measured by enzyme-linked immunosorbent assay (ELISA) using clear flat-bottom 384-well plates (Thermo Fisher Scientific, 464718), which were coated overnight at room temperature with 50 ng/well of EDIII protein in Phosphate-Buffered Saline (PBS). After washing with PBS-T (PBS + 0.05% Tween-20), plates were blocked with PBS, 1mM EDTA, 0.05% Tween-20 and 1% Bovine Serum Albumin (BSA) for 2 h at room temperature. After washing with PBS-T, serum or monoclonal antibodies (prediluted in PBS-T) were added to the plate and incubated for 1 h at room temperature. After washing with PBS-T, secondary sheep anti-human-IgG conjugated with horseradish Peroxidase (HRP; VWR, NA933; diluted 1:5000 in PBS-T) was added to the plate and incubated for an additional 1 h at room temperature. After the final washes, TMB (3,3',5,5'-Tetramethylbenzidine) substrate (Thermo Fisher Scientific, 34021) was added to develop the plate, 1M sulfuric acid was added to stop the reaction, and the plates read at 450nm at the luminometer (BioTek, EPOCH2 microplate reader). Sera were serially diluted 3-fold, starting at 1:50 (Figure 1A). Recombinant monoclonal antibodies were serially diluted 3-fold, starting at 1.5 μ g/mL (Figure 2A). Nonlinear regression analysis was used to determine the half-maximal effective concentration for binding (EC₅₀) using Prism software (GraphPad), as previously detailed.⁹³ For testing the crossreactivity to the EDIII of tick- or *Aedes*

mosquito-borne Orthoflaviviruses, recombinant monoclonal antibodies were tested at 1.5 $\mu\text{g}/\text{mL}$ according to the protocol described above. An anti-SARS CoV-2 monoclonal antibody, hr2.024 was used as isotype control.⁸⁰ The broadly reactive EDIII antibody P003 was used as positive control.⁵²

For the polyreactivity assay, 384-well plates were coated overnight at room temperature with 10 $\mu\text{g}/\text{mL}$ for ssDNA, dsDNA, LPS, and KLH, and with 5 $\mu\text{g}/\text{mL}$ for insulin in PBS. After washing with PBS +0.001% Tween-20-0.001% (PBSt), plates were blocked with PBS 1x, 0.5M EDTA, 0.0005% Tween-20 and 2.5% BSAIb for 2 h at room temperature. After washing with PBSt, monoclonal antibodies were added and incubated for 1 h. After washing with the same buffer, the same secondary antibody was added and incubated for 1 h. The development and stop of the reaction were performed as described above. ED38 polyreactive antibody⁸² was used as a positive control and ZIKV antibody Z021 was used as negative control.³²

To determine the half-life of monoclonal antibody W010, human IgG concentrations were quantified in mouse serum samples by ELISA following previously published protocols. Briefly, high-binding, half-area 96-well microtiter plates (Nunc) were coated overnight at 4°C with neutravidin (3 $\mu\text{g}/\text{mL}$ in PBS). After washing in PBS-T buffer, plates were incubated with biotinylated goat anti-human IgG antibodies for 1 h (5 $\mu\text{g}/\text{mL}$) and blocked for 2 h with PBS/2% bovine serum albumin. Serially diluted serum samples were incubated for 1 h, followed by incubation with horseradish peroxidase-conjugated anti-human IgG (1:10,000). Plates were developed using the TMB two-component peroxidase substrate kit (KPL), and reactions were stopped with the addition of 1 M sulfuric acid. Absorbance at 450 nm was immediately recorded using a SpectraMax Plus spectrophotometer (Molecular Devices), and background absorbance from negative control samples was subtracted. Antibody half-life was calculated as described.⁹⁴

For the detection of serum autoantibodies against IFN- α 2 (Figure S1P), an in-house developed ELISA was performed using the same protocol as for the EDIII, except that plates were coated with 5 $\mu\text{g}/\text{mL}$ of human IFN- α 2 recombinant protein and serum reactivity was tested starting at 1:50 dilution, followed by 4-fold serial dilutions.

Determination of Tm

The melting temperature (Tm) of IgG antibodies (diluted in PBS) was determined using the ThermalShift Assay kit (ThermoFisher) on a QuantiStudio 6 flex thermal cycler, following manufacturer recommendations. Raw fluorescent values from quadruplicate samples were analyzed using the TSA craft tool as described⁹⁵ and Tm (°C) are reported.

Detection of anti-IFN- α 2 and anti-IFN- ω autoantibodies

Detection of anti-IFN- α 2 and anti-IFN- ω IgG (Figures 1D–1F) was performed using a multiplexed bead-based assay essentially as described previously.^{46,48} Serum was tested at a 1:100 dilution. For each sample, median fluorescence intensity (MFI) values from the IFN- α 2 or IFN- ω coated beads were obtained and made relative to the corresponding MFI value obtained from non-coated empty beads (fold over empty, FOE). Values were then normalized to in-house plate controls, and sera exhibiting normalized FOE values >5 SDs above the mean values obtained from a negative sample panel were considered positive for the specific anti-IFN- α 2 or - ω IgG. Neutralizing anti-IFN- α 2 or anti-IFN- ω activity was determined using the highly sensitive AIR cell system.⁴⁹ Briefly, AIR cells were seeded at 30,000 cells per well in a 96-well plate in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin (Gibco, 15140-122), and incubated overnight at 37°C, 5% CO₂. Serum samples were diluted 1:20 in OptiMEM (Optimized Minimal Essential Medium; Gibco, 31985070) containing 0.01 ng/mL IFN- α 2 (Novus Biologicals, NBP2-34971) or 0.02 ng/mL IFN- ω (Novus Biologicals, NBP2-35893), together with a live-cell Renilla luciferase substrate (EnduRen, Promega, E6481; 1:10,000), and incubated for 1 hour with constant shaking at 600 rpm. The serum:IFN-I mixtures were then used to stimulate AIR cells for 16 hours at 37°C, 5% CO₂. Renilla luciferase activity was determined using a PerkinElmer EnVision plate reader (EV2104) and obtained luciferase values were normalized to in-house plate controls. Thresholds for positive neutralization activity were defined as a 75% reduction of IFN-I-induced luciferase value relative to the average value obtained from a negative sample panel.

Single cell sorting

Isolation of WNV_{EDIII} memory B cells was performed as described.³⁴ Briefly, the Pan B-cell isolation kit (Miltenyi Biotec, no. 130-101-638) and LS magnetic columns (Miltenyi Biotec; 130-042-401) were used to enrich B cells from PBMCs of WND convalescent individuals according to manufacturer's instructions. The enriched B cells were subsequently incubated for 30 min on ice in FACS buffer (PBS, 2% FBS) with biotinylated WNV_{EDIII} conjugated to streptavidin-PE (eBioscience, Cat. No. 12-4317-87), with biotinylated WNV_{EDIII} conjugated to streptavidin-Alexa Fluor 647 (BioLegend; Cat. No. 405237) and with biotinylated Ovalbumin (Sigma-Aldrich; A5503-1G) conjugated to streptavidin-BV711 (BD Horizon; 563262). The incubation was performed in the presence of anti-CD14-APC-eFluor 780 (Thermo Fisher Scientific, 47-0149-42), anti-CD16-APC-eFluor 780 (Thermo Fisher Scientific, 47-0168-41), anti-CD8-APC-eFluor 780 (Invitrogen, 47-0086-41), anti-CD3-APC-eFluor 780 (Thermo Fisher Scientific, 47-0037-42), Zombie NIR (BioLegend, 423105) and anti-CD20-PE-Cy7 (BD Biosciences, 335828). Zombie NIR⁺CD14⁺CD16⁺CD3⁺CD8⁺CD20⁺Ova⁺WNV_{EDIII}-PE⁺WNV_{EDIII}-AF647⁺ B cells were then single-cell sorted into 96-well plates containing lysis buffer (0.5 $\times$ PBS, 10 mM dithiothreitol, and RNasin ribonuclease inhibitors; Promega, N2615) using the FACSsymphony S6 cell sorter (BD Biosciences) and the analysis performed with FlowJo software. Sorted cells were snap-frozen on dry ice and then stored at -80°C.

Antibody gene sequencing, cloning and expression

Single-cell RNA was reverse-transcribed (SuperScript III Reverse Transcriptase, Invitrogen 18080-044) and the resulting cDNA stored at -80°C until PCR-amplification of the variable Ig heavy and light genes by nested PCR reactions and Sanger sequencing,

as detailed previously.^{84,96} Amplicons were next used for Sequence- and Ligation-Independent Cloning (SLIC) into antibody and Fab expression vectors.³² Recombinant monoclonal antibodies and Fabs were produced, purified and quality controlled as previously described.³² The E16 humanized sequence was retrieved from the literature⁴¹ and the antibody produced in-house starting from synthetic DNA (GenScript). Human one-armed IgG1 bispecific antibody was engineered using the variable regions of W010 or E16 and SARS-CoV-2 antibody sd1.040 in the CrossMAb format. Briefly, four expression plasmids were co-transfected at a 1:1:1:1 ratio, and antibodies were purified from cell supernatants as previously described.⁸⁰

Sequence analysis

Antibody sequences were analysed using in-house R and Perl scripts (available on GitHub; <https://github.com/stratust/igpipeline>). Briefly, sequences were annotated using Igblast tool v2; the sequences from the same cell were paired and assigned clonotypes based on V and J genes. Additionally, nucleotide somatic hypermutation and CDR3 length were analysed. Hypermutation analysis was based on the closest germlines sequences in IgBlast tool.

Surface plasmon resonance (SPR) and epitope mapping

SPR assays were performed on a Biacore 8K instrument (Cytiva) at 25 °C, using 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) pH 7.4, 150 mM NaCl, 3 mM EDTA and 0.005% Tween-20 as running buffer. Kinetics parameters were determined as described.⁹⁷ Antibodies were immobilized (150 nM) on the surface of CM5 chips (Cytiva) and increasing concentrations of the WNV_{EDIII} protein (15.62, 31.25, 62.5, 125, 250, 500 nM) were injected using a single-cycle kinetics setting; binding responses were corrected for unspecific binding and buffer responses. Curve fitting, data analysis and graphs were done with Biacore Insight Evaluation Software v.2.0.15.12933. Antibody competition experiments were performed to assess whether the antibodies recognize overlapping epitopes on the WNV_{EDIII} protein. Antibodies (W007, W009, W010, W014, W037, W048, W049) were immobilized as before and the WNV_{EDIII} protein was injected at a saturating concentration (500 nM). Immediately after, the second antibody was injected (150 nM) followed by a regeneration step with H₃PO₄ (0.1 M) to remove the EDIII protein and the second antibody. Additional cycles of injection of the EDIII protein, antibodies and H₃PO₄ were subsequently done. Curve fitting, data analysis and graphs were done with Biacore Insight Evaluation Software v.2.0.15.12933.

Plasmids for the production of reporter virus particles (RVPs)

A WNV subgenomic replicon-expressing plasmid encoding Renilla luciferase (pWNVII-Rep-REN-IB; obtained from Ted Pierson at National Institutes of Health, NIH)⁹⁸; was modified by replacing Renilla by the NanoLuc luciferase coding sequence to generate pWNVII-Rep-NanoLuc. More in detail: plasmid pHIV-1_{NL4-3}ΔEnv-NanoLuc⁹⁹ was used as template for PCR with primers 5'-GTACGCGTATGGTCTTCACACTCGAAGA-3' and 5'-GAAGTTACGCGCCGTCGCCAGAATGCGTTCGCACA-3', while a second PCR was performed on plasmid pWNVII-Rep-REN-IB using primers 5'-GAACGCATTCTGGCGACGGCGCGTAACCTTCGACCT-3' and 5'-CACCCACAGAGTGTGGGT-3'. Upon purification, the two PCR products were combined by overlap PCR and ligated into plasmid pWNVII-Rep-REN-IB, previously digested with MluI (New England Biolabs, R3198S) and DraIII (New England Biolabs, R3510S), resulting in plasmid pWNVII-Rep-NanoLuc. The plasmid expressing the WNV CprME was previously reported (pWNV/TX02/CprME).¹⁰⁰ For the generation of plasmids expressing the CprME of JEV (SA-14 strain; GenBank: M55506.1), MVEV (MK6684 strain; GenBank: KF751869.1), SLEV (Parton strain; GenBank: EF158070.1) and USUV (Vienna 2001 strain; GenBank: NC_006551), synthetic DNA segments with the corresponding CprME coding sequences were amplified by PCR prior to cloning into the NotI/PacI sites of pPOWV-LB-CprME³⁴ resulting in plasmids pJEV-SA-14-CprME, pMVEV-MK6684-CprME, pSLEV-Parton-CprME and pUSUV-Vienna2001-CprME, respectively. The plasmids used to express the CprME of ZIKV and TBEV were previously reported.^{32,34}

RVP production

6-well plates were seeded with 7.5x10⁵ HEK293T cells/well. After 24 h cells were co-transfected using Lipofectamine 2000 (Invitrogen, 11668019) with a mix of pWNV-NanoLuc and CprME expressing plasmids at a 1:3 ratio.³⁴ After 5 h of incubation at 37°C, the medium was replaced with DMEM high glucose GlutaMAX (Gibco) supplemented with 3% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. For the next 72 h, every 24 h RVPs were harvested, filtered (0.22 µm), aliquoted and frozen at -80°C, and the medium replaced. Infectivity was determined by titration (see [Figure S1C](#)).

RVP neutralization assays

96-well plates were seeded with 1.5x10⁴ Huh-7.5 cells/well in 100 µL DMEM high glucose GlutaMAX (Gibco) supplemented with 10% FBS, 1% sodium pyruvate, 1% non-essential amino acids, 100 U/mL penicillin and 100 µg/mL streptomycin. After 24 h, the antibody containing samples were diluted with Ba-1 medium (Medium 199 (Gibco), 1% BSAIb, 100 U/mL penicillin and 100 µg/mL streptomycin), then 200 µL diluted serum or antibody were combined with 200 µL diluted RVPs diluted in Ba-1 medium and incubated for 1 h at 37°C. 100 µL of the mix were then added in triplicate to the plate and incubated overnight at 37°C. The supernatant was next aspirated, the cells washed with PBS, and 50 µL lysis buffer added (Promega, E1531). The Nano-Glo Luciferase Assay System (Promega, N1120) with GloMax Discover System reader (Promega) were used to measure the NanoLuc luciferase activity of the lysate. Serum was diluted 1:100 for the WNV_{RVP} neutralization screening ([Figure 1B](#)). Otherwise, it was diluted 1:150 and then serially 1:3. Recombinant monoclonal antibodies were tested starting with 10 µg/mL (WNV RVPs) or 1 µg/mL (JEV, MVEV, SLEV and USUV

RVPs), Fab molecules at 6.7 $\mu\text{g/mL}$, and then serially diluted 1:3. Molar Neutralization Ratio (MNR) was calculated as described.¹⁰¹ Nonlinear regression analysis was used to determine the NT_{50} and IC_{50} values using Prism software (GraphPad). In the cross-neutralization screening against the panel of orthoflavivirus RVPs (Figure S6A), recombinant antibodies were tested at a single concentration of 500 ng/mL using the protocol described above, and the results compared to no antibody control.

Plaque assay

The viral titers were determined using a plaque assay. Viruses were serially diluted in DMEM and incubated with A549 cells (2×10^5 per well) for 3 hours at 37 °C in 5% CO_2 . Cells were then overlaid with DMEM containing 1.5% carboxymethylcellulose and incubated under the same conditions for either 4 days (WNV lineage I and II) or 5 days (JEV, MVEV and SLEV) prior to washing with PBS, and staining with naphthalene black solution to visualize plaques. The viral titers were calculated and expressed as plaque-forming units per milliliter (PFU/mL).

Virus neutralization assay and immunofluorescent staining

All tested monoclonal antibodies were initially diluted in DMEM medium to a starting concentration of 10 $\mu\text{g/mL}$ and subsequently subjected to a 2-fold serial dilution in 96-well plates. Following dilution, 50 PFU of WNV, lineage I or II, were added to each well and the mixtures were incubated for 90 minutes at 37 °C in 5% CO_2 . After incubation, 3×10^4 A549 cells were added to each well, and the plates were further incubated under the same conditions. Cytopathic effects were monitored five days post-infection using an inverted microscope. Virus neutralization assay was performed in octuplicates for each concentration and the result expressed as the percentage of neutralization over no-antibody control for each concentration (Figure 3B).

A similar procedure was employed for the fluorescence-based neutralization assay (Figure S3A). Three (JEV, MVEV and SLEV) or four days (WNV lineage I and II) post-infection, cells were fixed with cold acetone/methanol (1:1) and subsequently blocked with 10% FBS. Cells were then incubated with a mouse anti-flavivirus antibody in 1:250 dilution (Sigma, MAB10216), washed three times with PBS-T, and incubated with a fluorescently labelled secondary goat anti-mouse antibody in 1:500 dilution (Invitrogen, A32723). After three additional washes with PBS-T, cell nuclei were stained using DAPI at a 1:2000 dilution (Sigma, D9542). Fluorescence images were acquired using the ImageXpress Pico automated imaging system and CellReporterXpress software (Molecular Devices, USA).

Selection of antibody-resistant WNV variants

To select for antibody-resistant variants of WNV 13-104, the virus was serially passaged in the presence of increasing concentrations of the monoclonal antibody W010 in A549 cells. Briefly, W010 was diluted in DMEM and incubated with 5000 PFU of WNV 13-104 for 90 minutes at 37 °C in a 24-well plate. Following incubation, 2×10^5 A549 cells were added to each well. In parallel, wild-type virus was passaged under identical conditions without the addition of antibody as a control. Five days post-infection, supernatants from wells displaying CPE were harvested and used for subsequent passages with progressively higher concentrations of W010. After RNA isolation (QIAGEN, 52904), viral genomic RNA was quantified at each passage using quantitative reverse transcription PCR with the Advanced Kit for West Nile Virus (Genesig, Z-Path-WNV-EASY) and Lyophilised OneStep qRT-PCR Master Mix (Oasig, Z-oasig-onestep-150), according to the manufacturers' instructions. The virus envelope (E) gene was sequenced with primers WNV_1A (CCTGGTGGCACCAGCATACAGC) + WNV_1B (GTGGTGCTTCCAGCACTGCTCC) and WNV_2A (GCGAAGTCCTTCCTGGTTCACCGA) + WNV_2B (CACAGCCTGTGTCTAGCATGGACG) and analyzed from viral populations passaged in the presence of 50 ng/mL W010 to assess mutations associated with antibody resistance.

The plasmid to express the mutant WNV RVP N368T was obtained by site-directed mutagenesis of the plasmid pWNV/TX02/CPME using the QuikChange Multi Site-Directed Mutagenesis following manufacturer instructions.

Structure determination via X-ray crystallography

W037 Fab and W014 Fab were expressed in Expi293F cells, and subsequently purified via Nickel-NTA (nitriloacetic acid) as previously described.³⁴ W010 IgG was expressed in Expi293F cells, purified by Protein A (ProA) affinity chromatography and cleaved with 1.5% papain to generate Fab fragments. Fabs were subsequently isolated from Fc components or uncleaved IgG by purification over a ProA column. All Fabs were purified via size exclusion chromatography (SEC) over a Superdex 200 10/300 column against 1x TBS (20 mM Tris pH 8.0, 150 mM NaCl). Fab-WNV EDIII complexes were generated by combining WNV EDIII with Fab at a 2:1 ratio overnight at room temperature, followed by purification via SEC using a Superdex 200 10/300 column to separate complex from excess WNV EDIII. Fractions attributed to the complex were analyzed by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) prior to pooling and concentrating to 15 mg/mL.

Crystallization trials were set up using the sitting drop vapor diffusion method by mixing equal volumes of complex and reservoir using a Mosquito LCP liquid handling robot (SPT LabTech) and commercially available 96-well crystallization screens (Hampton Research). Crystals were grown at 22 °C and observed in multiple conditions. The single crystal used for structure determination of W037 Fab-WNV EDIII was obtained in 0.2 M magnesium chloride hexahydrate, 20% w/v polyethylene glycol (PEG) 3,350. The single crystal used for structure determination of W010 Fab-WNV EDIII was obtained in 20% w/v PEG4,000, 0.2M disodium malonate. The single crystal used for structure determination of W014 Fab-WNV EDIII was obtained in 20% w/v 2-propanol, 20% w/v PEGME (polyethylene glycol monomethyl ether) 2000, 0.1 MES (2-(n-morpholino) ethanesulfonic acid). The single crystal used for structure determination of W049 Fab-WNV EDIII was obtained in 12% w/v PEG3,350 and 0.1M disodium DL-malate. All crystals were cryo-protected in a solution matching the reservoir and 20% glycerol and then cryocooled in liquid nitrogen.

All X-ray diffraction data was collected at the Stanford Synchrotron Radiation Lightsources on beamline 12-2 with a Pilatus 6M detector at a wavelength of 0.979 Å and temperature of 100K. Data from a single crystal was indexed and integrated in XDS¹⁰² and merged using AIMLESS in CCP4.¹⁰³ Structures were determined using molecular replacement in Phenix PHASER¹⁰⁴ using each of the following individual chains as search models with CDR3 loops trimmed: WNV EDIII for all structures (PDB 6UTE); W037 Fab V_H (PDB 6Z3K) and V_L (PDB 6WOZ); W010 Fab V_H (PDB 6DWC) and V_L (7BEI); W014 Fab V_H (PDB 5GGU) and V_L (PDB 7BEI); W049 Fab V_H and V_L (PDB 6DWC). Coordinates were refined using iterative rounds of automated and interactive refinement in Phenix¹⁰⁵ and Coot,⁸⁸ respectively. Statistics for the final models can be found in [Table S4](#).

Structural analyses

Complementarity-determining regions (CDRs) and somatic mutations were annotated using IMGT (International ImMunoGeneTics information system) V-QUEST.¹⁰⁶ Structural visualizations were generated with ChimeraX.¹⁰⁷ Buried surface area calculations were performed via the PDBePISA web server.¹⁰⁸ Residue contacts were defined as atom-atom distances under 4 Å between different chains. Hydrogen bonds were identified using a distance cutoff of 3.5 Å and an angle criterion with an A–D–H angle exceeding 90°. Root-mean-square deviation (RMSD) values were calculated using ChimeraX. Antibody residue numbering follows the Kabat scheme.

Mouse protection experiments

The animal experiments adhered to all relevant European Union guidelines for animal research and were conducted in accordance with Czech national law (Animal Welfare Act No. 246/1992 Coll.), ensuring the ethical use and protection of experimental animals. The study protocol was approved by the Ministry of Agriculture of the Czech Republic (permit nos. 22006/2016-MZe-17214 and MZE-23932/2024-13143).

Five experimental groups ($n=12$ per group) were established. Mice were inoculated intraperitoneally with 200 μ L PBS containing monoclonal antibody W010 (30 μ g/mouse) either one day before infection or 1, 3, or 5 days post-infection. Isotype control was antibody 10-1074.⁸¹ On the day of infection, all mice were challenged subcutaneously with a lethal dose of WNV strain 13-104 (10^4 PFU/mouse). Mouse protection experiments with W014 were conducted as with W010, except that mice were treated only at day -1 and with 300 μ g of either W014 ($n=10$) or isotype control 10-1074 ($n=5$).

For mouse protection experiments in the presence of anti-IFNAR antibodies, four experimental groups ($n=6$ per group) were established. Three groups were inoculated intraperitoneally with 200 μ L PBS containing 100 μ g anti-IFNAR antibody (BioXCell, #BP0241), while the control group received 200 μ L of PBS alone. The anti-IFNAR antibody or PBS alone were administered one day before infection and then every other day, for a total of three doses. On the day of infection, the control group and one of the anti-IFNAR-treated groups were intraperitoneally injected with 200 μ L of PBS containing an isotype control monoclonal antibody. The other 2 anti-IFNAR-treated groups received 200 μ L of PBS containing 200 μ g of W010 on either day 0 or 1 after infection. All mice were challenged subcutaneously with a lethal dose (10^4 PFU per mouse) of WNV strain 13-104.

The animals were closely monitored for health status, clinical symptoms, and survival for up to 18 or 28 days. Survival rates were analyzed by log rank Mantel-Cox test in GraphPad Prism.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical tests were as indicated throughout the methods and in the figures or their legends. The analyses were performed with Prism software (version 10.6.1; GraphPad), unless otherwise noted.

Supplemental information

Analysis of West Nile disease convalescents identifies human monoclonal antibodies protective against West Nile and related orthoflaviviruses

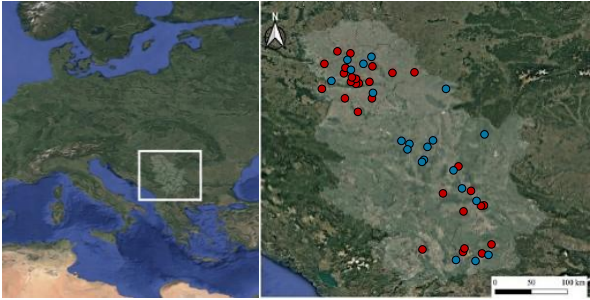
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Figure S1

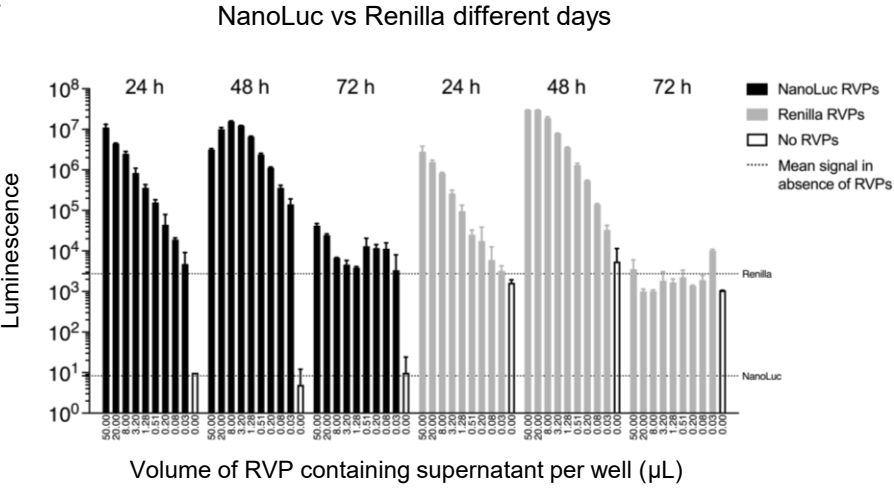
A

Cohort	Age	Gender			
		Male		Female	
		n	%	n	%
WND	59.10 years (CI 95%: 55.145 – 63.055)	45	65.21	24	34.78
WNF	42.33 years (CI 95%: 31.99 - 52.68)	1	33.33	2	66.66

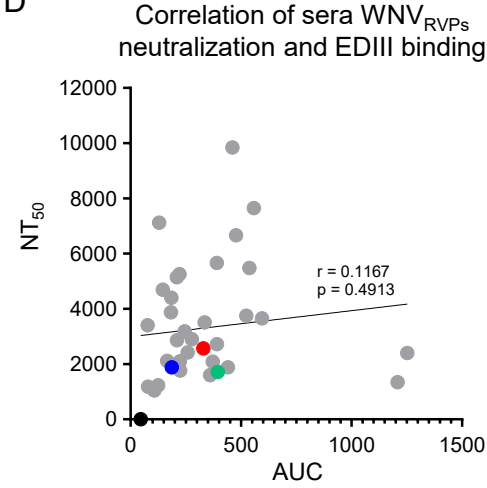
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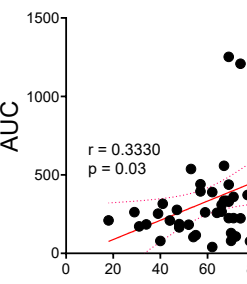
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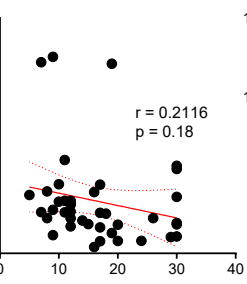
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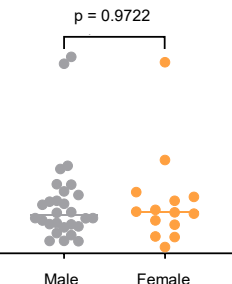
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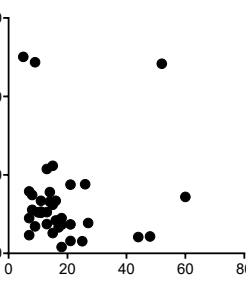
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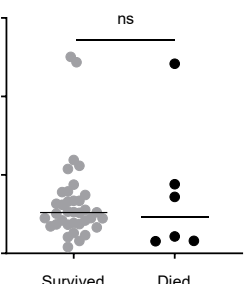
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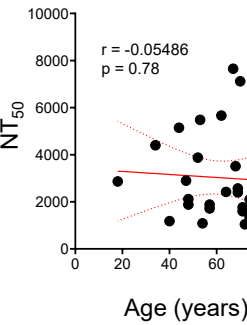
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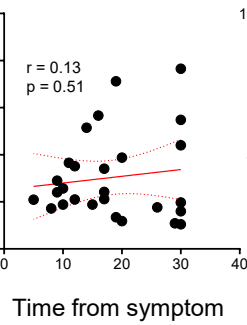
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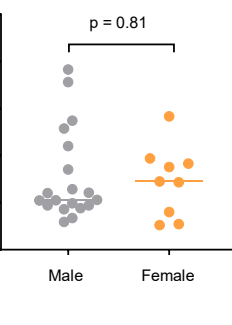
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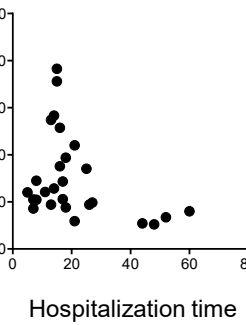
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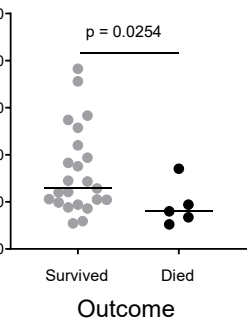
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M



N



O

anti-IFN-α2 autoantibodies					anti-IFN-ω autoantibodies				
Age group	Serbian cohort	General population	p-value	Significantly higher?	Age group	Serbian cohort	General population	p-value	Significantly higher?
<50	0 / 8 (0%)	8 / 2710 (0.30%)	—	No	<50	0 / 8 (0%)	40 / 4446 (0.90%)	—	No
50–60	2 / 7 (28.6%)	3 / 1736 (0.17%)	< 0.001	Yes	50–60	1 / 7 (14.3%)	13 / 1736 (0.75%)	0.004	Yes
60–70	4 / 12 (33.3%)	14 / 2475 (0.57%)	< 0.001	Yes	60–70	4 / 12 (33.3%)	12 / 2475 (0.48%)	< 0.001	Yes
70–80	1 / 10 (10.0%)	29 / 1790 (1.62%)	0.15	No	70–80	1 / 10 (10.0%)	29 / 1790 (1.62%)	0.15	No
80–90	0 / 2 (0%)	79 / 1580 (5.00%)	—	No	80–90	0 / 2 (0%)	56 / 1580 (3.54%)	—	No
50–80	7 / 29 (24.1%)	46 / 6001 (0.766%)	< 0.001	Yes	50–80	6 / 29 (20.6%)	54 / 6001 (0.899%)	< 0.001	Yes
P-values from two-sample, one-sided Fisher's exact tests									
"Significantly higher" defined as p < 0.05									
"—" indicates no test due to zero events in the cohort									

P

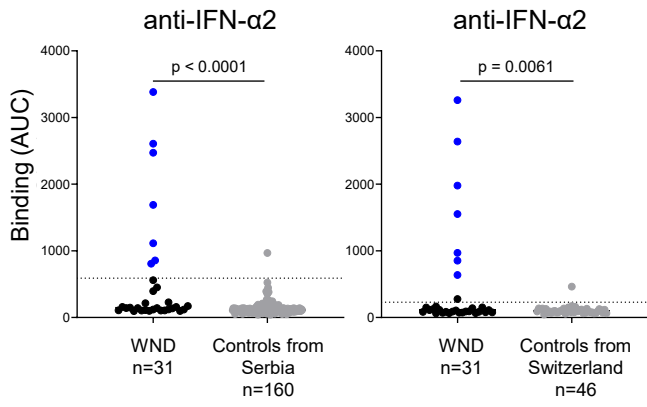


Figure S1. Hospitalized WND/WNF individuals show diverse serologic features. Related to main Figure 1.

(A) Demographic characteristics of the cohort. **(B)** Geographic distribution of the patients' residency. Case distribution shows the involvement of 11 Districts, with clusters in Central Serbia in 2022 and Northern Serbia in 2023. Blue dots indicate 2022 cases (with a dominant cluster near the city of Kragujevac, Šumadija District), while red dots represent the residency of the 2023 cases (with a dominant cluster near the city of Novi Sad). The map was generated with QGIS v3.12 (QGIS Development Team, 2020) starting from the GADM database (v4.1, July 2022, <https://gadm.org/>). **(C)** Comparison between NanoLuc and Renilla reporter virus particles (RVPs). WNV_{RVP} were produced side-by-side by transfecting packaging cells with a NanoLuc- or Renilla-expressing WNV replicon (see Materials and Methods). Different volumes of supernatant-containing RVPs harvested at 24h, 48h and 72h were added to Huh 7.5 cells and the luminescence measured from cell lysates. The lower background with NanoLuc results in improved signal-to-noise ratio. Dotted horizontal lines indicate the average luminescence that was measured in the absence of WNV_{RVP} . **(D)** Correlation between serum IgG binding to WNV_{EDIII} and NT_{50} values. AUC is Area Under the Curve of ELISA binding. Pearson correlation. **(E)** Correlation between serum IgG binding to WNV_{EDIII} and age at hospitalization. Pearson correlation. **(F)** Correlation between serum IgG binding to WNV_{EDIII} and time from symptoms onset to serum sampling. Pearson correlation. **(G)** Comparison of serum IgG binding to WNV_{EDIII} between samples from male and female participants. Unpaired two-tailed t-test. **(H)** Correlation between serum IgG binding to WNV_{EDIII} and days of hospitalization. Pearson correlation. **(I)** Comparison of serum IgG binding to WNV_{EDIII} between samples from survivors and non-survivors. Unpaired two-tailed t-test. **(J)** Correlation between WNV_{RVP} NT_{50} values and age at hospitalization. Pearson correlation. **(K)** Correlation between WNV_{RVP} NT_{50} values and time from symptoms onset to serum sampling. Pearson correlation. **(L)** Comparison of serum WNV_{RVP} NT_{50} values between samples from male and female participants. Unpaired two-tailed t-test. **(M)** Correlation between serum WNV_{RVP} NT_{50} values and days of hospitalization. Pearson correlation. **(N)** Comparison of WNV_{RVP} NT_{50} values between samples from survivors and non-survivors. Unpaired two-tailed t-test. **(O)** Prevalence of autoantibodies against type I IFNs in the WND cohort compared with available general population data.⁴⁹ One-sided Fisher's exact test. **(P)** Prevalence of autoantibodies against IFN- α 2 in the WND cohort compared with blood donor control samples from Serbia (left) and Switzerland (right), as determined by ELISA. AUC is Area Under the Curve. The threshold for positivity (dotted line) was determined set at 5 Standard Deviations of the signal of the controls. One-sided Fisher's exact test.

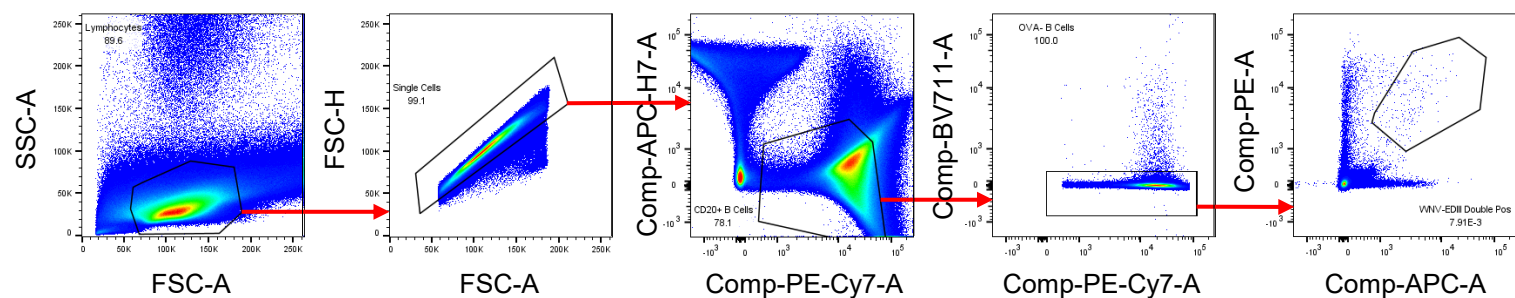
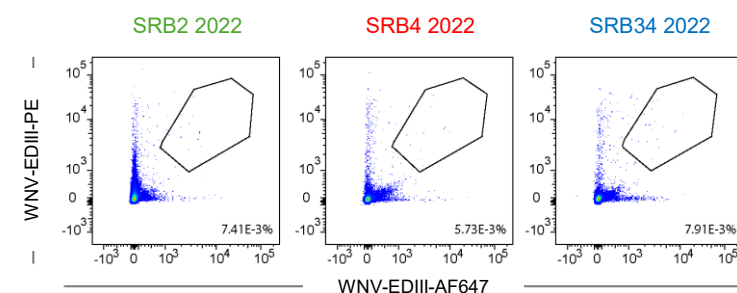
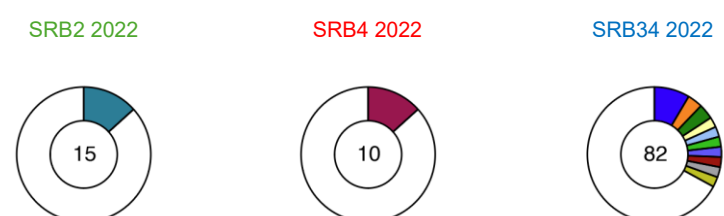
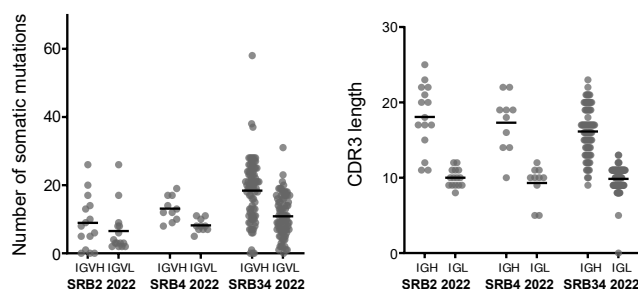
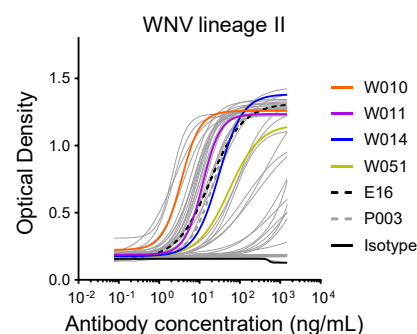
Figure S2**A****B****C****D****E**

Figure S2. WNV_{EDIII}-specific antibodies from WND convalescents display clonal diversity. Related to main Figure 2.

(A) Gating strategy. Forward and side scatter (FSC and SCC, respectively) were used to gate on lymphocytes. Dump channel included CD3, CD8, CD14, CD16 and a viability dye. CD20⁺ B cells binding to the WNV_{EDIII} coupled with PE and AF647 were isolated (see Methods for details). **(B)** Identification of WNV_{EDIII}-specific B cells. Flow cytometry plots show WNV_{EDIII}-specific B cells (gate) in three WND convalescents. Percentages refer to the gated double-positive cells. **(C)** Analysis of antibody sequences. Pie charts show the distribution of antibody sequences from sorted B cells (b). Coloured slices indicate clonally related sequences, while singlets are in white. In the center of the pie is the total number of sequences derived from each convalescent individual. **(D)** Number of antibody V gene somatic nucleotide mutations and amino acid length of the complementarity-determining region 3 (CDR3). **(E)** Monoclonal antibody binding to the EDIII of WNV lineage II. ELISA curves show the binding of 44 monoclonal antibodies to the EDIII of protein of WNV lineage II. Positive control is the broadly reactive antibody P003;⁷⁹ humanized mouse antibody E16⁴¹ is shown for comparison. Representative of two independent experiments.

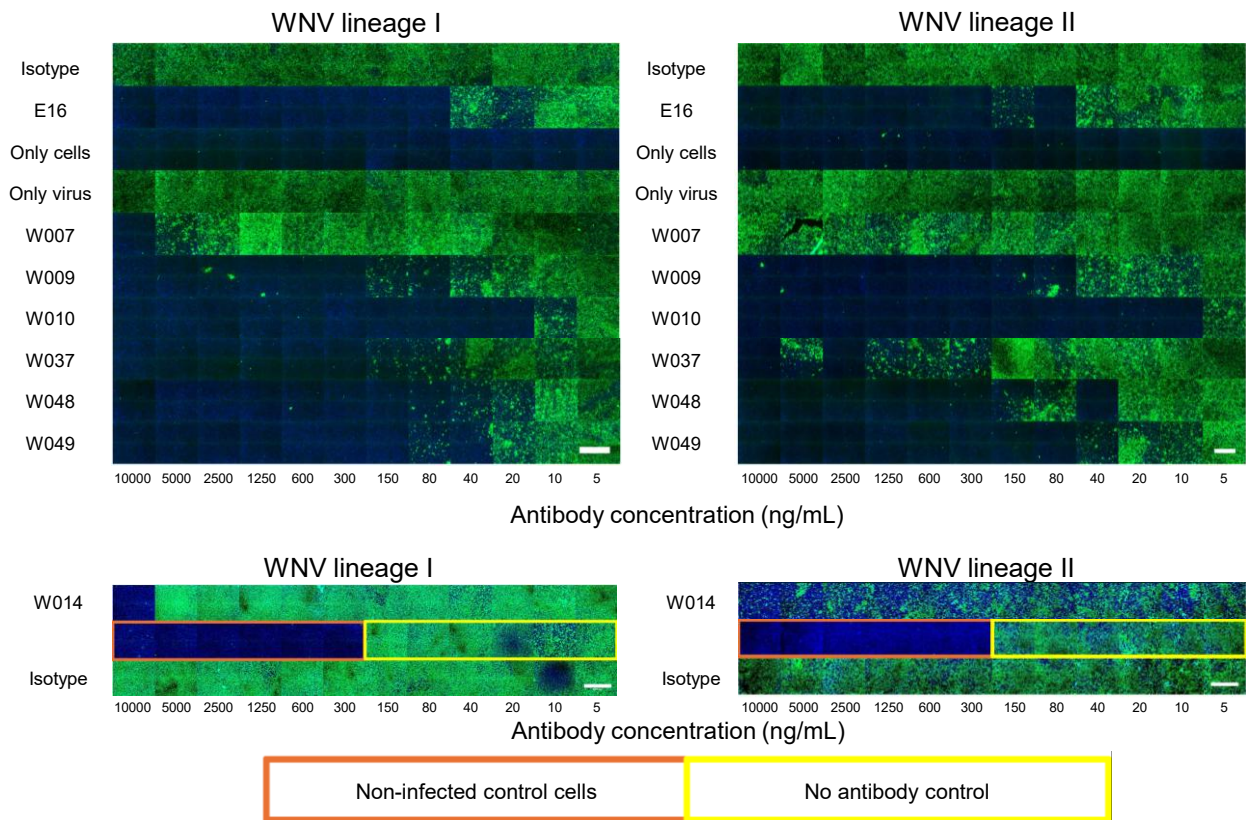
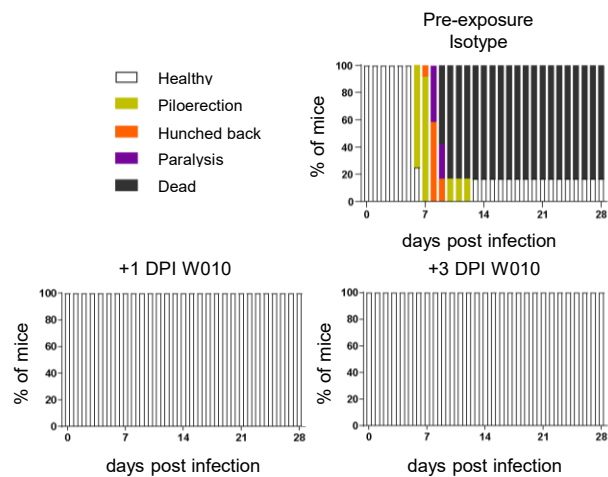
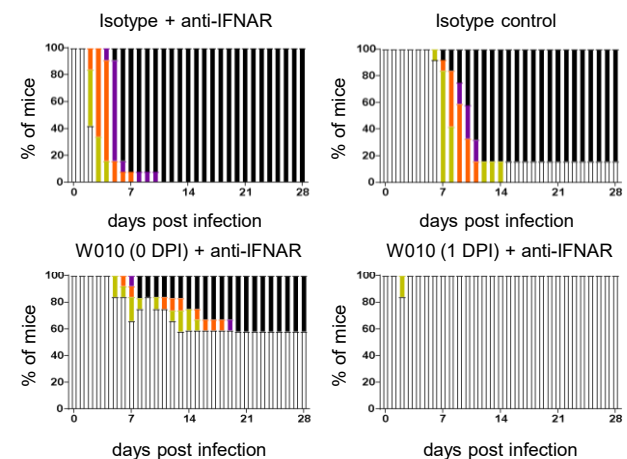
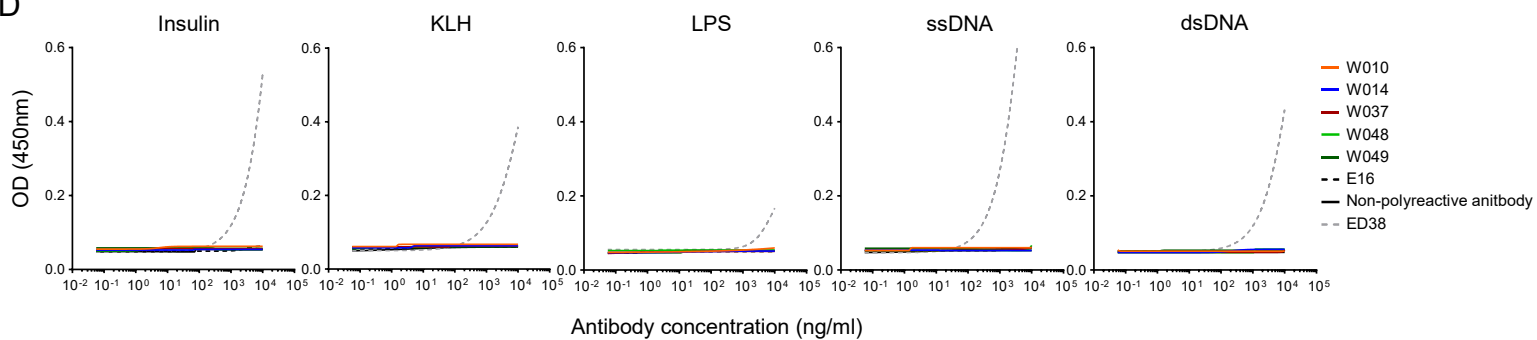
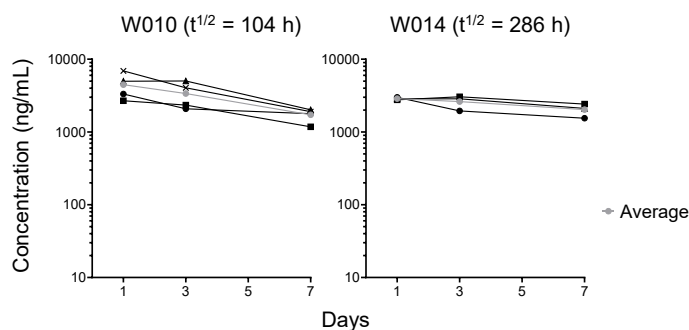
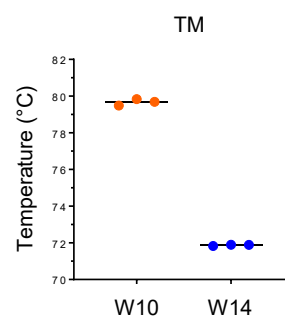
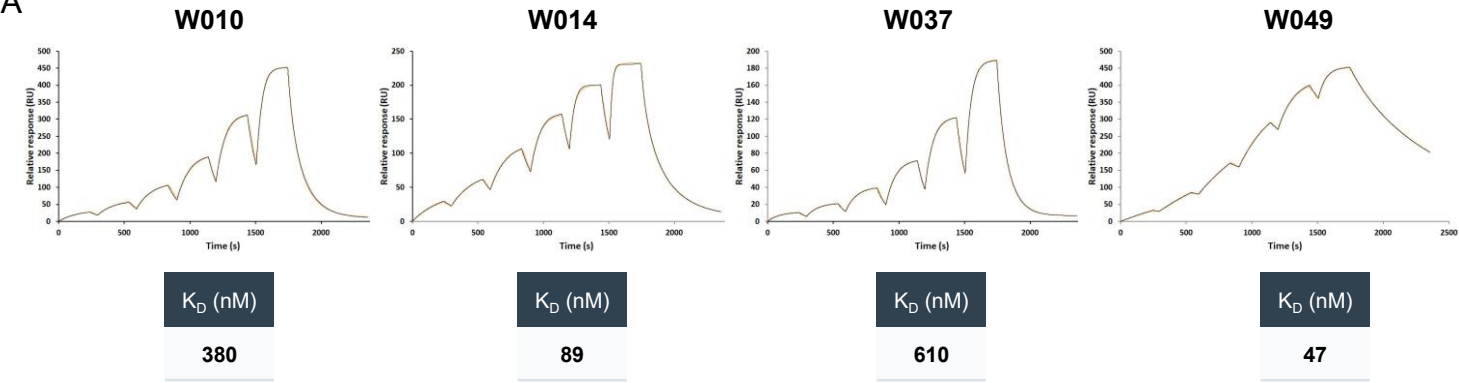
Figure S3**A****B****C****D****E****F**

Figure S3. W010 shows in vitro and in vivo efficacy, and features supporting its clinical development. Related to main Figure 3.

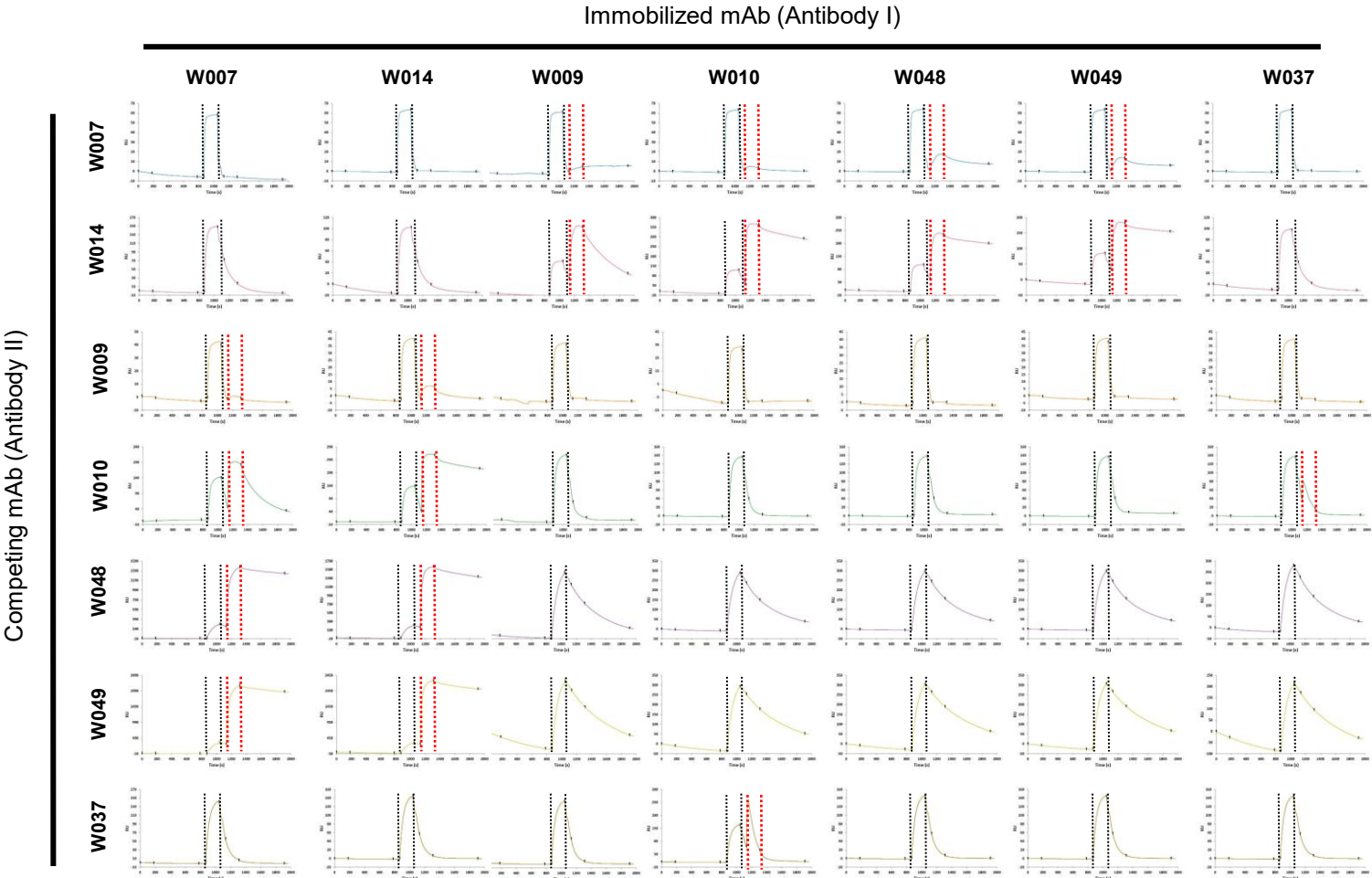
(A) Representative qualitative immunofluorescence microscopy images are shown for neutralization of WNV lineage I and II by the indicated monoclonal antibodies. Green is viral antigen, and blue is cell nuclei (4',6-diamidino-2-phenylindole (DAPI)). Scale bar indicates 500 μm . **(B)** Mouse symptoms over time after WNV challenge. DPI is days post infection. Related to Figure 3C. **(C)** Symptoms over time after WNV challenge in mice treated with anti-IFNAR. Related to Figure 3D. **(D)** Antibodies' polyreactivity. Monoclonal antibodies binding to insulin, keyhole limpet hemocyanin (KLH), lipopolysaccharyd (LPS), single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) was evaluated by ELISA. ED38 is a polyreactive monoclonal antibody used as positive control.⁸⁰ **(E)** Half-life of W010 and W014 in humanized mice⁵¹ upon intravenous injection (50 μg). **(F)** Melting temperature values of WNV antibodies.

Figure S4

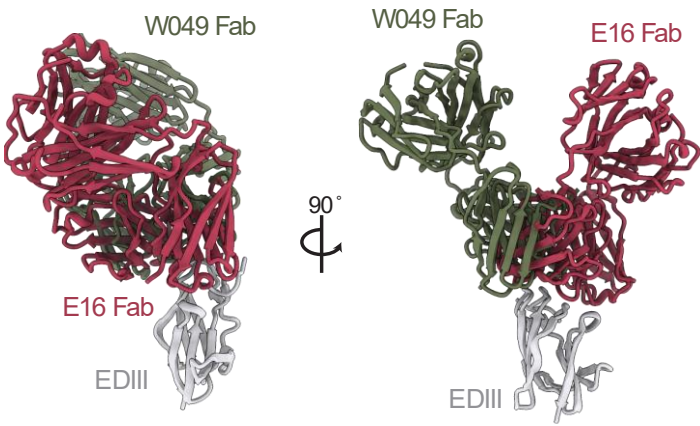
A



B



C



D

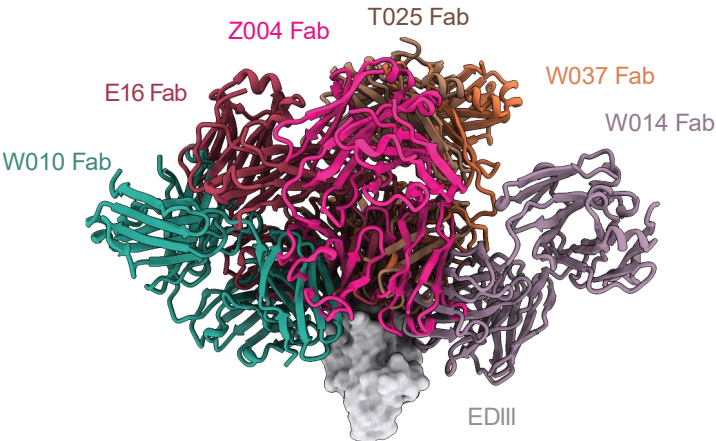


Figure S4. WNV_{EDIII}-specific monoclonal antibodies show different binding kinetics and epitope specificities. Related to main Figure 4.

(A) Representative Surface Plasmon Resonance plots of the assay to determine the antibody's binding to the EDIII antigen (KD values). **(B)** Competition of binding experiments identify antibodies binding to non-overlapping epitopes on the EDIII of WNV. Dotted black lines indicate EDIII binding to the immobilized antibody, while dotted red lines show the association of the non-competing antibody (or lack thereof when the lines are absent). **(C)** Superposition of crystal structures of WNV_{EDIII} in complex with W049 and E16 (PDB 1ZTX). **(D)** Superposition of crystal structures of WNV_{EDIII} in complex with W010, W037, W014, and E16 Fabs and canonical lateral ridge antibodies against Zika virus (Z004 Fab, PDB 6UTA) and tick-borne encephalitis virus (T025 Fab, PDB 7LSE).

Figure S5

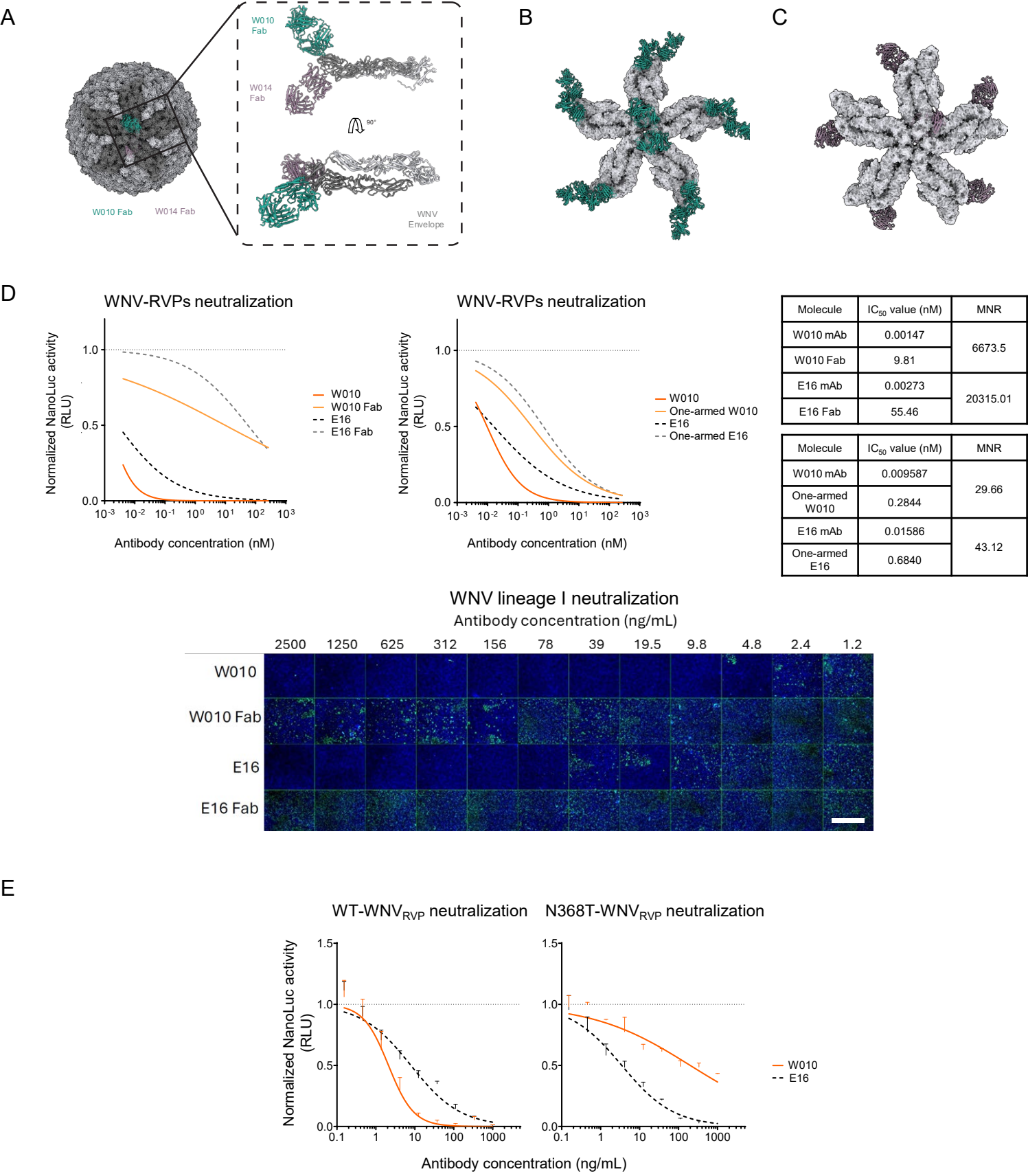
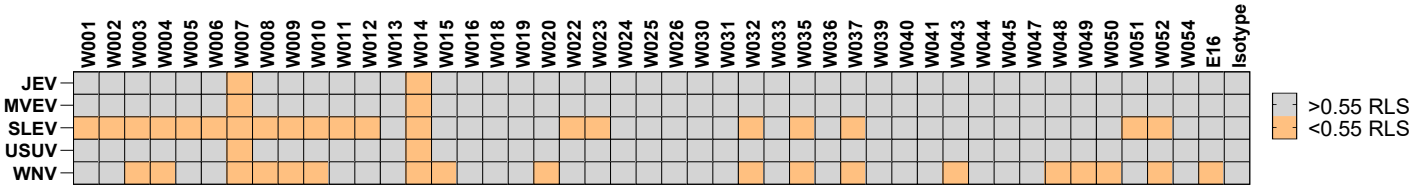


Figure S5. W010 and W014 target non-overlapping epitopes on WNV_{EDIII}. Related to main Figure 5.

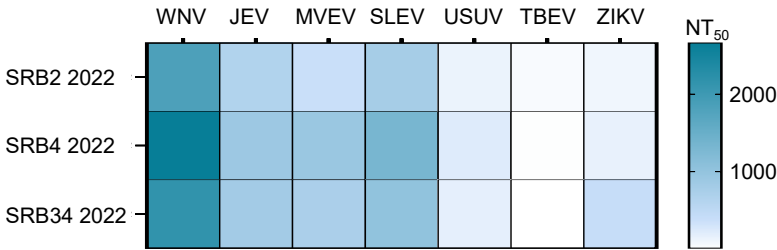
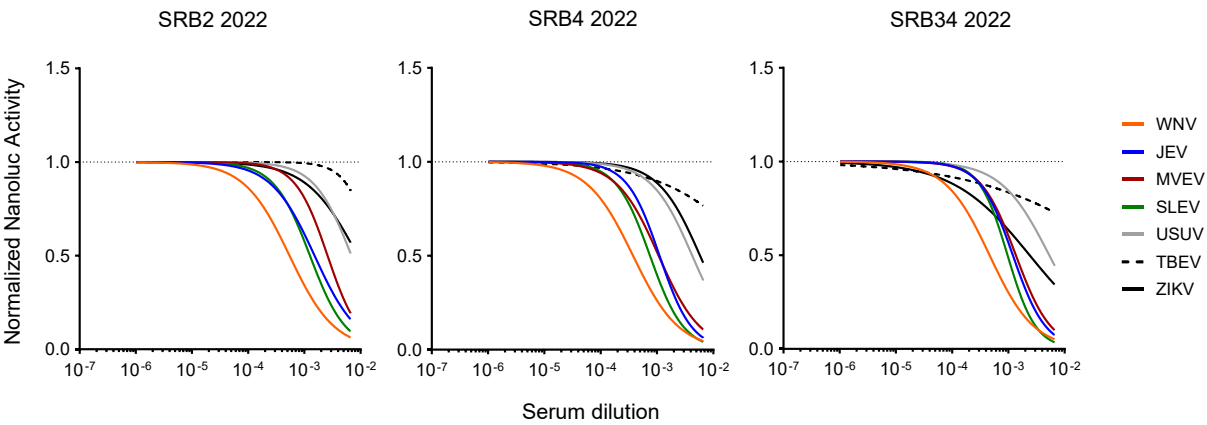
(A) W010-EDIII and W014-EDIII were aligned with mature WNV virion (PDB 7KVA), with associated RMSD values of 0.52 and 0.55 respectively. **(B and C)** Modeling of W010 (B) and W014 (C) Fab fragments binding to their respective EDIII epitopes at the 5-fold symmetry axis of the WNV. **(D)** Neutralization of WNV (either RVPs, top, or authentic virus, bottom) by W010, comparing IgG1 to either Fab or to one-armed IgG1 (one arm W010 or E16, the other isotype), as indicated. Scale bar indicates 500 μ m. MNR is Molar Neutralization Ratio.⁸¹ **(E)** Neutralization of wild type (WT) and mutant N368T WNV_{RVP} by W010 and E16. NanoLuc activity in the presence of serially diluted antibodies relative to no-antibody control is shown. Mean $\pm$ SD of triplicates. Representative of two experiments.

Figure S6

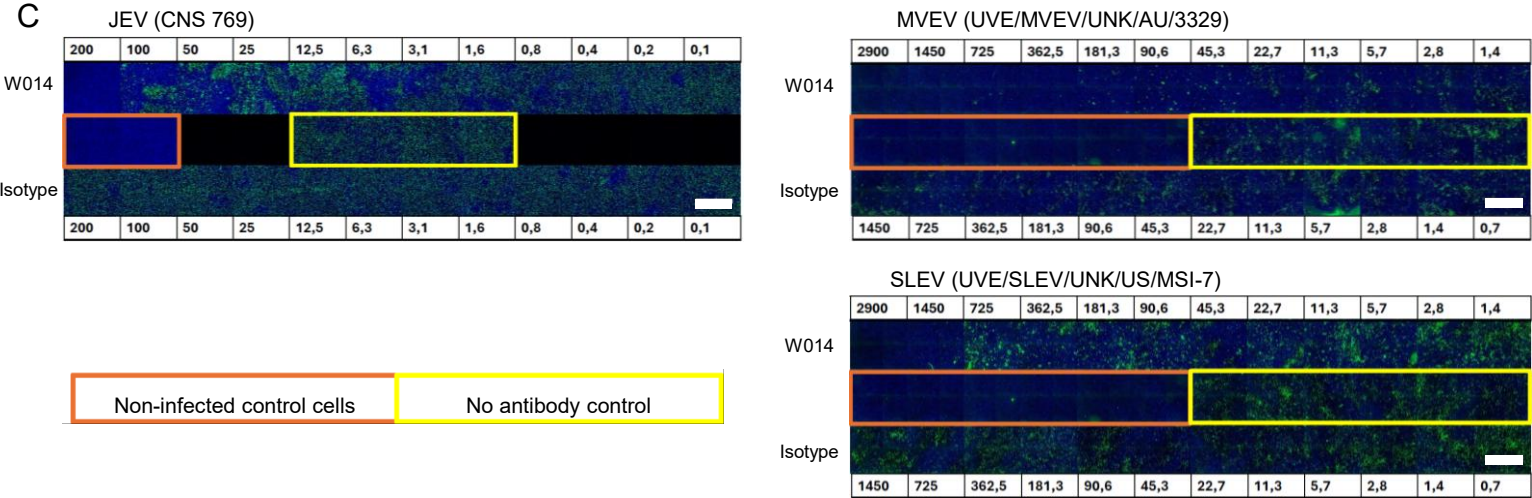
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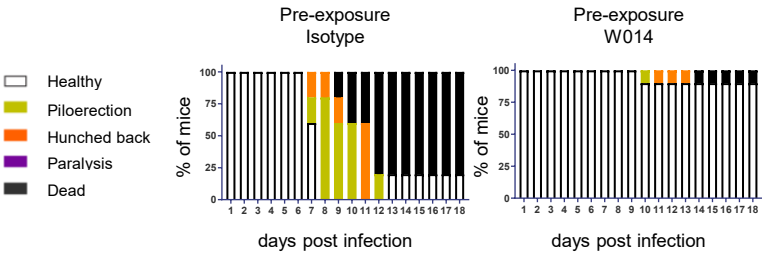


Figure S6. Monoclonal antibodies and sera cross-neutralize WNV and related orthoflaviviruses. Related to main Figure 6.

(A) Monoclonal antibodies were analysed at a single dilution (500 ng/mL) for the ability to neutralize RVPs corresponding to WNV, JEV, MVEV, SLEV and USUV. NanoLuc activity relative to “no-antibody” control is shown. Orange represents the antibodies for which the NanoLuc activity of the RVPs was quenched by more than 55%. A single experiment, average of triplicate wells. RLS is relative luciferase signal over no-antibody control. **(B)** Sera cross-neutralization. Sera from WND convalescents, from which monoclonal antibodies were derived, cross-neutralize RVPs corresponding to members of the JE serocomplex, TBEV and ZIKV. Neutralization curves are shown on top, heatmap with NT₅₀ values is at the bottom. **(C)** Representative qualitative immunofluorescence microscopy images are shown for neutralization of JEV, MVEV and SLEV by W014 and isotype control. Concentrations are expressed in µg/mL. Green is viral antigen, and blue is cell nuclei (DAPI). Scale bar indicates 500 µm. **(D)** Mouse symptoms over time after WNV challenge in the presence of W014 or isotype control. DPI is days post infection. Related to Figure 6G.

Table S1. Characteristics of study participants (n=72). Related to Figures 1 and S1.

Participant ID	Gender (M/F)	Age at diagnosis (years)	Residency (place)	Suspected disease (WNF/WND)	Clinical diagnosis	High serum neutralization in the screening (yes/no)	Anti-type I IFN neutralization (IFNa2, IFNo, both)	Time from symptom onset to serum sampling (days)	Duration of hospitalization (days)	Outcome (survived/died)	Clinical Center
SRB1 2022	M	57	Kragujevac	WND	Viral meningoencephalitis (WNV)	yes	both	10	26	Died	Clinical Center Kragujevac
SRB2 2022	M	57	Kragujevac	WND	Viral meningitis (WNV)	yes	n.d.	8	7	Survived	Clinical Center Kragujevac
SRB3 2022	M	77	Arandelovac	WND	Viral meningoencephalitis (WNV)	yes	n.d.	5	8	Survived	Clinical Center Kragujevac
SRB4 2022	M	69	Topola	WND	Viral meningoencephalitis (WNV)	yes	n.d.	10	14	Survived	Clinical Center Kragujevac
SRB5 2022	F	66	Velika Plana	WND	Viral meningoencephalitis (WNV)	no	n.d.	12	11	Survived	Clinical Center Kragujevac
SRB6 2022	F	68	Arandelovac	WND	Viral meningoencephalitis (WNV)	yes	n.d.	12	16	Survived	Clinical Center Kragujevac
SRB7 2022	M	67	Topola	WND	Viral meningoencephalitis (WNV)	no	n.d.	11	11	Survived	Clinical Center Kragujevac
SRB8 2022	M	66	Kragujevac	WND	Viral meningoencephalitis (WNV)	no	n.d.	12	15	Survived	Clinical Center Kragujevac
SRB9 2022	F	59	Rača	WND	Viral meningoencephalitis (WNV)	no	n.d.	11	10	Survived	Clinical Center Kragujevac
SRB10 2022	F	81	Kragujevac	WND	Viral meningoencephalitis (WNV)	no	n.d.	7	9	Survived	Clinical Center Kragujevac
SRB11 2022	M	31	Topola	WND	Viral meningoencephalitis (WNV)	no	n.d.	12	9	Survived	Clinical Center Kragujevac
SRB12 2022	M	69	Paraćin	WND	Viral meningoencephalitis (WNV)	no	n.d.	8	7	Survived	Clinical Center Kragujevac
SRB14 2022	M	50	Kragujevac	WND	Viral meningoencephalitis	yes	NT	9	15	Survived	Clinical Center Kragujevac
SRB15 2022	M	64	Kragujevac	WND	Viral meningoencephalitis (WNV)	yes	both	17	11	Survived	Clinical Center Kragujevac
SRB16 2022	F	47	Kragujevac	WNF	West Nile fever	yes	NT	9	8	Survived	Clinical Center Kragujevac
SRB17 2022	M	29	Arandelovac	WND	Viral meningoencephalitis (WNV)	no	n.d.	7	13	Survived	Clinical Center Kragujevac
SRB18 2022	M	55	Topola	WND	Viral meningoencephalitis (WNV)	no	n.d.	9	7	Survived	Clinical Center Kragujevac
SRB21 2022	M	64	Donji Ljubeš	WND	Status febrilis, encephalopathia	yes	NT	12	12	Survived	Clinical Center Niš
SRB22 2022	M	49	Vrtište	WND	Viral encephalitis	no	NT	11	ND	ND	Clinical Center Niš
SRB23 2022	F	39	Niš	WNF	West Nile fever	no	NT	18	ND	Survived	Clinical Center Niš
SRB24 2022	M	41	Niš	WNF	West Nile fever	no	NT	19	ND	Survived	Clinical Center Niš
SRB25 2022	M	78	Gnjilane	WND	Viral encephalitis	no	NT	5	ND	ND	Clinical Center Niš
SRB26 2022	F	63	Majdanpek	WND	Viral encephalitis	no	NT	4	ND	ND	Clinical Center Niš
SRB27 2022	M	73	Vranjska banja	WND	Viral encephalitis	no	NT	6	ND	ND	Clinical Center Niš
SRB28 2022	M	66	Bujanovac	WND	Viral encephalitis	no	NT	5	ND	ND	Clinical Center Niš
SRB29 2022	M	68	Bačka Palanka	WND	Viral encephalitis	no	NT	4	10	Survived	Clinical Center Novi Sad
SRB30 2022	F	76	Bečej	WND	Viral encephalitis	no	NT	9	ND	ND	Clinical Center Novi Sad
SRB31 2022	F	54	Nadailj	WND	Viral encephalitis (WNV)	yes	n.d.	29	44	Survived	Clinical Center Novi Sad
SRB32 2022	M	63	Stepanovićevo	WND	Viral encephalitis	no	NT	7	ND	ND	Clinical Center Novi Sad
SRB33 2022	M	48	Novi Sad	WND	Viral encephalitis (WNV)	yes	n.d.	17	17	Survived	Clinical Center Novi Sad
SRB34 2022	M	48	Indija	WND	Viral encephalitis (WNV)	yes	n.d.	15	13	Survived	Clinical Center Novi Sad
SRB35 2022	F	62	Novi Sad	WND	cephalitis (WNV) and non-Hodgkin lym	no	n.d.	16	18	Survived	Clinical Center Novi Sad
SRB36 2022	M	40	Vrbas	WND	Viral encephalitis (WNV)	yes	n.d.	20	21	Survived	Clinical Center Novi Sad
SRB37 2022	M	29	Novi Sad	WND	Viral encephalitis	no	NT	17	ND	ND	Clinical Center Novi Sad
SRB38 2022	F	72	Golubinci	WND	Viral encephalitis (WNV)	yes	n.d.	30	48	Died	Clinical Center Novi Sad
SRB1 2023	M	53	Grabovci	WND	WNV encephalitis	yes	IFNa2	30	13	Survived	Clinical Center Novi Sad
SRB2 2023	F	62	Sečanj	WND	WNV encephalitis	yes	n.d.	16	14	Survived	Clinical Center Novi Sad
SRB3 2023	F	52	Šid	WND	WNV encephalitis	yes	n.d.	20	18	Survived	Clinical Center Novi Sad
SRB4 2023	M	70	Odžaci	WND	WNV encephalitis	no	n.d.	24	21	Died	Clinical Center Novi Sad
SRB5 2023	M	44	Čurug	WND	WNV encephalitis	yes	n.d.	14	16	Survived	Clinical Center Novi Sad
SRB6 2023	F	81	Šremska Kamenica	WND	WNV encephalitis	yes	n.d.	11	ND	Survived	Clinical Center Novi Sad
SRB7 2023	M	67	Ravno Selo	WND	WNV encephalitis	yes	both	30	15	Survived	Clinical Center Novi Sad
SRB8 2023	M	88	Futog	WND	Encephalitis viralis non-specificata	yes	NT	ND	14	Survived	Clinical Center Novi Sad
SRB9 2023	M	79	Ledinci	WND	WNV encephalitis	yes	n.d.	30	27	Survived	Clinical Center Novi Sad
SRB10 2023	M	74	Golubinci	WND	WNV encephalitis	yes	n.d.	12	7	Survived	Clinical Center Novi Sad
SRB11 2023	M	71	Novi Sad	WND	WNV encephalitis	yes	n.d.	26	18	Survived	Clinical Center Novi Sad
SRB12 2023	M	70	Novi Sad	WND	WNV encephalitis	yes	n.d.	19	15	Survived	Clinical Center Novi Sad
SRB13 2023	M	34	Feketić	WND	Meningoencephalitis WNV	yes	n.d.	30	21	Survived	Clinical Center Novi Sad
SRB14 2023	M	69	Prokuplje	WND	WNV encephalitis	yes	both	9	5	Survived	Clinical Center Novi Sad
SRB15 2023	F	18	Nova Crvenka	WND	Meningoencephalitis WNV	yes	n.d.	ND	17	Survived	Clinical Center Novi Sad
SRB16 2023	M	78	Kovilj	WND	Encephalitis acuta	yes	NT	15	17	Survived	Clinical Center Novi Sad
SRB17 2023	M	64	Zrenjanin	WND	Encephalitis viralis non-specificata	yes	NT	20	20	Survived	Clinical Center Novi Sad
SRB18 2023	M	62	Rumenka	WND	Encephalitis viralis non-specificata	yes	NT	25	28	Survived	Clinical Center Novi Sad
SRB19 2023	M	72	Bački Petrovac	WND	Encephalomyelitis	yes	NT	16	16	Survived	Clinical Center Novi Sad
SRB20 2023	M	78	Šid	WND	WNV encephalitis	yes	n.d.	17	25	Died	Clinical Center Novi Sad
SRB21 2023	M	74	Futog	WND	WNV encephalitis	yes	both	19	52	Died	Clinical Center Novi Sad
SRB22 2023	M	69	Šremska Mitrovica	WND	WNV encephalitis	no	both	17	21	Survived	Clinical Center Novi Sad
SRB23 2023	F	71	Šremska Kamenica	WND	WNV encephalitis	yes	n.d.	30	60	Died	Clinical Center Novi Sad
SRB24 2023	F	35	Niš	WND	Encephalitis viralis non-specificata	no	NT	5	ND	ND	Clinical Center Niš
SRB25 2023	F	75	Niš	WND	Encephalitis viralis non-specificata	no	NT	5	ND	ND	Clinical Center Niš
SRB26 2023	F	55	Vranje	WND	Encephalitis viralis non-specificata	no	NT	5	ND	ND	Clinical Center Niš
SRB27 2023	F	49	Niš	WND	Encephalitis viralis non-specificata	no	NT	8	ND	ND	Clinical Center Niš
SRB28 2023	M	24	Kostadince	WND	Encephalitis viralis non-specificata	no	NT	4	ND	ND	Clinical Center Niš
SRB29 2023	F	64	Sastavar	WND	Encephalitis viralis non-specificata	no	NT	3	ND	ND	Clinical Center Niš
SRB30 2023	M	77	Niš	WND	Meningoencephalitis acuta	no	NT	5	ND	ND	Clinical Center Niš
SRB31 2023	M	30	Glogovac	WND	Encephalitis viralis non-specificata	no	NT	5	ND	ND	Clinical Center Niš
SRB32 2023	F	59	Niš	WND	Encephalopathia	no	NT	2	ND	ND	Clinical Center Niš
SRB33 2023	F	28	Niš	WND	Encephalitis viralis non-specificata	no	NT	5	ND	ND	Clinical Center Niš
SRB34 2023	F	28	Aleksinac	WND	Encephalitis viralis non-specificata	yes	NT	8	20	Survived	Clinical Center Niš
SRB35 2023	M	18	Vladičin Han	WND	Encephalitis viralis non-specificata	no	NT	10	ND	ND	Clinical Center Niš
SRB36 2023	F	61	Doljane	WND	Encephalitis viralis non-specificata	no	NT	7	ND	ND	Clinical Center Niš
SRB37 2023	F	57	Popovac	WND	Encephalitis viralis non-specificata	no	NT	10	ND	ND	Clinical Center Niš

NT = Not Tested
n.d. = non detectable
ND = no data

Table S3. Effective and inhibitory concentrations of recombinantly expressed monoclonal antibodies. Related to Figures 3 and 6.

	EC ₅₀ (ng/mL)						IC ₅₀ (ng/mL) - RVPs					IC ₅₀ (ng/mL) - virus	
	WNV lin I - EDIII	WNV lin II - EDIII	JEV - EDIII	MVEV - EDIII	SLEV - EDIII	USUV - EDIII	WNV _{RVPs} lin I	JEV _{RVPs}	MVEV _{RVPs}	SLEV _{RVPs}	USUV _{RVPs}	WNV lin I	WNV lin II
W001													
W002													
W003													
W004	132	83.6											
W005	2.325	2.092											
W006													
W007							90.21	166.8	88.91	13.44	59.82	107.1	85.15
W008	20.6	10.66					15						
W009	4.281	2.204					782.1					24.71	18.23
W010	4.312	3.532					3.696					4.819	3.839
W011	9.398	11.88	17.27	203.1		17.89							
W012	6.792	3.747											
W013													
W014	42.22	30.06	31.41		15.94	17.84	102.2	43.61	27.77	4.327	36.27	772.9	142.9
W015	14.42	7.982					13.48						
W016	9.385	8.695											
W018	13.04	2.149					238.3						
W019	64.5	27.95											
W020													
W022	9.456	6.684					152.8						
W023	13.79	10.75					113.2						
W024													
W025	89.45	22.09											
W026													
W030													
W031													
W032										140.2			
W033													
W035	33.42	16.65											
W036													
W037	17.54	19.38					6.696					56.78	35.9
W039													
W040	173.6	129											
W041													
W043	8.256	7.048											
W044	23.33	11.12											
W045	18.46	8.676											
W047	4.958	7.519											
W048	4.603	6.017					34.25					14.14	13.09
W049	14.54	17.2					2.028					10.39	11.65
W050	97.35	86.75					14.11						
W051	53.47	53.68			17.19		1347		485.7	12.8			
W052													
W054	13.18	13.82					212.1						

Not-determined

Not-tested

Non-neutralizing

Table S4. Crystallographic data processing and refinement statistics. Related to Figures 4, 5, and 6.

		W010-WNV EDIII	W014-WNV EDIII	W037-WNV EDIII	W049-WNV EDIII
PDB ID		9ZRM	9ZRN	9ZRO	9ZRP
Data collection ^a					
Space group		P1	P 1 21 1	P 21 21 2	P 21 21 21
Unit cell (Å)		39.9 56.8 67.8	41.1 122.1 54.1	74.9 158.9 52.4	97.179 112.691 129.888
α, β, γ (°)		66.5 89.4 70.2	90 106.331 90	90 90 90	90 90 90
Wavelength (Å)		0.97946	0.97946	0.97946	0.97946
Resolution (Å)		37.14 - 1.8 (1.864 - 1.8)	39.54 - 2.1 (2.175 - 2.1)	37.76 - 1.4 (1.45 - 1.4)	39.55 - 2.5 (2.56 - 2.5)
Unique Reflections		42869 (4320)	29372 (2825)	122938 (11699)	93009 (6498)
Completeness (%)		90.80 (91.79)	98.35 (93.76)	99.38 (95.62)	97.86 (96.99)
Redundancy		3.9 (3.8)	3.6 (3.1)	6.7 (6.3)	6.9 (7)
CC _{1/2} (%)		99.7 (94.3)	99.1 (51.7)	99.9 (62.1)	99.6 (48.7)
<I/σI>		11.9 (3.8)	5.42 (1.38)	12.29 (2.13)	8.7 (1.2)
Mosaicity (°)		0.12	0.17	0.09	0.15
R _{merge} (%)		6.6 (30.3)	8.579 (60.53)	2.812 (38.43)	14.7 (95.1)
R _{pim} (%)		3.8 (17.9)	8.579 (60.53)	2.812 (38.43)	9.1(63.5)
Wilson <i>B</i> -factor		17.6	28.09	13.75	55.18
Refinement and Validation					
Resolution (Å)		37.14 - 1.8	39.54 - 2.1	37.76 - 1.4	39.55 - 2.5
Number of atoms					
	Protein	4016	4096	4016	8365
	Ligand	0	0	0	
	Waters	480	367	788	176
R _{work} /R _{free} (%)		17.7/21.6	19.8/25.1	18.3/21.1	20.5/26.2
R.m.s. deviations					
	Bond lengths (Å)	0.004	0.024	0.013	0.008
	Bond angles (°)	0.78	1	1.3	0.98
MolProbity score		0.98	1.46	1.06	1.78
Clashscore (all atom)		1.88	3.9	1.76	4.75
Poor rotamers (%)		0.44	1.3	0.88	1.64
Ramachandran plot					
	Favored (%)	97.89	96.6	97.3	94.52
	Allowed (%)	2.1	3.7	2.67	5.38
	Disallowed (%)	0	0	0	0.09
Average <i>B</i> -factor (Å)		22.36	32.13	19.9	58.41
^a Numbers in parentheses correspond to the highest resolution shell					

5. Other contributions

During my PhD studies, I contributed to the execution of several additional projects. Some of these already resulted in publications, while others are still ongoing. Although some of these works are not included as independent thesis chapters, they are scientifically and/or methodologically connected to the topics explored here and they provided me with a valuable experience, broadened my scientific perspective, and allowed me to build connections with other researchers in the field.

1. Bianchini, F., Crivelli, V., Abernathy, M. E., Guerra, C., Palus, M., Muri, J., ... **Cervantes Rincon, T.**, ... & Robbiani, D. F. (2023). Human neutralizing antibodies to cold linear epitopes and subdomain 1 of the SARS-CoV-2 spike glycoprotein. *Science Immunology*. <https://doi.org/10.1126/sciimmunol.ade0958>
2. Svoboda, P., Haviernik, J., Bednar, P., Matkovic, M., **Cervantes Rincón, T.**, Keeffe, J., ... & Ruzek, D. (2023). A combination of two resistance mechanisms is critical for tick-borne encephalitis virus escape from a broadly neutralizing human antibody. *Cell reports*. <https://doi.org/10.1016/j.celrep.2023.113149>
3. Mijatović, D., Radevska, A. M., Jakimovski, D., Popović-Dragonjić, L., Popovska Jovičić, B., Gavrilović, J., ... **Cervantes Rincon, T.**, ... & Banović, P. (2026). Underrecognized Tick-Borne Encephalitis in Serbia: Evidence from Patients with Suspected West Nile Virus Neuroinvasive Disease. *Pathogens*. <https://doi.org/10.3390/pathogens15060587>
4. Clinical development of monoclonal antibody TBE025; ongoing.
5. Characterization of cross-neutralizing antibodies against tick-borne (T056/T058 mAbs) and mosquito-borne (Z015/Z039 mAbs); ongoing.

My contribution to these works is related to antibody production, purification and characterization by ELISA, assay development, RVPs neutralization and epitope mapping.

6. Discussion

Orthoflaviviruses represent a diverse group of arthropod-borne viruses, capable of causing severe neurological disease in humans. Members of this group include mosquito-borne viruses such as WNV, JEV, and ZIKV, as well as tick-borne viruses such as POWV and TBEV. Despite the differences in ecological niches and transmission vectors, these viruses share a highly conserved virion architecture in which the envelope (E) glycoprotein is at the outer surface of the virus particle and mediates receptor attachment and membrane fusion. Because of its exposure and critical role in viral entry, the E protein constitutes one of the main targets of neutralizing antibody responses and therefore represents an important antigen for therapeutic antibody discovery and vaccine development.

The results presented in this work show the complexity of antibody responses targeting the EDIII protein, which has been characterized as a major determinant of potent neutralizing antibodies. Antibodies targeting this region often show strong protective activity in animal models and sometimes also cross-neutralization among related viruses.^{291,302} Structural and functional analyses performed here reinforce the central role of EDIII in being a valuable target of orthoflavivirus neutralization by antibodies while also revealing substantial diversity in the mechanisms by which antibodies recognize this domain.

A notable observation is the discovery of EDIII antibodies (P002 and P003) capable of recognizing orthoflaviviruses belonging to distinct ecological groups, including both mosquito-borne and tick-borne viruses. Such cross-reactivity is somewhat unexpected given the antigenic divergence between the orthoflavivirus groups. Cross-reactive antibodies have previously been described for conserved epitopes within the EDII fusion loop or the prM protein; however, these antibodies typically exhibit limited neutralization potency.^{12,348,349} In contrast, antibodies recognizing EDIII are generally more virus-specific but highly neutralizing, reflecting the structural variability of this region among orthoflaviviruses. The observation that anti-EDIII antibodies can recognize viruses from multiple serocomplexes suggests that conserved structural determinants within EDIII exist that are capable of eliciting broader recognition, although the neutralizing capacity of P002 and P003 is weak and limited to POWV.

The origin of such antibodies remains an open question. In orthoflavivirus-endemic regions, individuals are frequently exposed to multiple orthoflaviviruses over time, including DENV, ZIKV, WNV, and YFV, especially in tropical regions. Sequential infections with antigenically related viruses can drive extensive affinity maturation of B-cell responses and may lead to the generation of antibodies with broadened specificity profiles. Several studies have demonstrated that repeated orthoflavivirus exposure can reshape the antibody repertoire and expand cross-reactive memory B-cell populations.^{350,351} It is therefore possible that multiple exposures to different mosquito-borne orthoflaviviruses could induce antibodies capable of recognizing tick-borne orthoflaviviruses.

At the same time, the possibility that antigenically related yet uncharacterized orthoflaviviruses circulate in certain regions cannot be excluded. Orthoflavivirus diversity in nature is likely underestimated, particularly in ecosystems where vector surveillance remains limited. The presence of Ixodes ticks in tropical environments and sporadic historical reports of serological reactivity to tick-borne orthoflaviviruses suggest that additional members of this viral group may remain undiscovered.³⁵² If such viruses circulate at low levels or cause nonspecific febrile illness, or no symptoms at all, they may easily escape detection in areas where diseases such as dengue are highly prevalent.

Structural analyses of the antibodies described in this work provide important insight into the molecular basis of orthoflavivirus neutralization. Two principal epitope-targeting strategies appear to emerge. One class of antibodies recognizes the lateral ridge epitope of EDIII, a surface-exposed region that is accessible on the mature virion and has repeatedly been identified as a key neutralization hotspot.^{291,299,302,353,354} The lateral ridge is highly accessible across the virion surface and antibodies targeting this epitope often display strong neutralization potency. Structural modeling studies suggest that neutralization of orthoflaviviruses typically requires engagement of approximately thirty antibody molecules on the virion surface, a concept referred to as the neutralization threshold.³⁰⁸ Antibodies targeting exposed epitopes such as the lateral ridge can readily exceed this threshold, thereby efficiently blocking viral entry.

However, even antibodies recognizing similar residues can exhibit differences in neutralization potency. Structural and functional studies have shown that these differences are not only from the epitope but also from how antibodies engage the virion surface. Variations in binding orientation, angle of approach, and the capacity for multivalent engagement can influence neutralization efficacy.^{355,356} Subtle differences in antibody recognition can also determine whether epitopes located in sterically constrained or only transiently accessible regions of the virion are available for enhanced binding, thereby modulating neutralization activity.^{297,357} Structural analyses of highly potent antibodies further demonstrate that favorable geometric positioning and quaternary engagement on the virion surface can enhance neutralization efficiency, even among antibodies targeting overlapping regions.^{358,359} Together, these findings highlight the importance of considering not only epitope specificity but also the geometric and structural context of antibody binding when evaluating mechanisms of orthoflavivirus neutralization.

A second neutralizing epitope involves antibodies that recognize the CC' loop region of EDIII. This region contains residues that are relatively conserved within certain orthoflavivirus serocomplexes and may therefore support broader cross-reactivity. However, structural studies indicate that the CC' epitope is partially buried in the mature virion conformation.^{357,360} As a result, antibodies recognizing this region may rely on transient structural rearrangements of the E protein that occur during viral entry. In the endosome, due to lower pH, the E protein undergoes a dramatic conformational transition from a homodimeric to a homotrimeric state, potentially exposing otherwise hidden

epitopes. Antibodies capable of recognizing these intermediate states may therefore neutralize infection by interfering with the fusion process rather than by blocking initial attachment to target cells.^{361,362}

Another aspect highlighted by our findings is the complex relationship between innate antiviral defenses and adaptive antibody responses. IFN-I play a critical role in controlling orthoflavivirus replication during early infection.¹⁴ Experimental models of orthoflavivirus infection lacking components of the IFN signaling pathway, including IFNAR, STAT1, or IRF7, exhibit markedly increased susceptibility to neuroinvasive disease.³⁶³⁻³⁶⁷ Similarly, individuals with impaired IFN signaling-due to the presence of anti-IFN neutralizing autoantibodies-may experience severe West Nile disease combined with higher viral loads, which could in turn lead to increased antigen exposure and stronger humoral responses, as we show. However, elevated antibody titers do not translate into improved neutralization capacity, indicating that antibody quality is a more important determinant of protective immunity than antibody quantity alone.

Some of the monoclonal antibodies characterized in this work have translational potential. Passive immunotherapy using mAbs represents a promising approach for preventing or treating viral infections, including orthoflavivirus infections, particularly in individuals at high risk of severe disease. Neutralizing antibodies can provide immediate protection and can be administered prophylactically during outbreaks or therapeutically following exposure. Advances in antibody engineering have further improved the clinical potential of such therapies by enabling the development of extended half-life antibodies and Fc-engineered variants of anti-NS1 antibodies with altered effector functions.³⁶⁸⁻³⁷⁴

Nevertheless, mAbs and vaccines should be regarded as complementary rather than competing strategies. mAbs provide rapid, targeted protection but typically require repeated administration and may be expensive to produce and administer. Vaccines, by contrast, aim to induce long-lasting immune memory and provide population-level protection. However, vaccine design for orthoflaviviruses presents several challenges. Antigenic similarity between viruses can lead to cross-reactive antibodies that lack strong neutralizing activity and may contribute to ADE, a phenomenon well documented in DENV infection.^{28,183,313,375,376} Furthermore, the structural heterogeneity of orthoflavivirus particles, including the presence of immature, partially mature, and mature virions, complicates the accessibility of antibodies to protective epitopes.^{13,81,377,378}

Structural information obtained from antibody Fab-virus complexes provides powerful guidance for structure-based vaccine design. Rational antigen engineering strategies aim to focus the immune response toward protective epitopes while minimizing responses against non-neutralizing regions.^{379,380} For example, targeted mutations can be introduced to stabilize specific conformations of EDIII or to disrupt immunodominant epitopes that do not contribute to neutralization.^{381,382} Similarly, engineered glycosylation sites may be used to mask undesirable antigenic surfaces and redirect antibody responses toward conserved neutralization determinants.³⁸³⁻³⁸⁵ Stabilization of specific structural states of the E protein may also enable the presentation of otherwise transient

epitopes, such as those exposed during membrane fusion.^{320,386} Additionally, multivalent presentation of repetitive surface-expressed antigens, such as through virus-like particles or nanoparticle scaffolds, can significantly enhance immunogenicity by promoting efficient B cell receptor cross-linking and robust germinal center responses.³⁸⁷⁻³⁹⁰

Recent advances in germline-targeting immunogen design further expand these possibilities. By analyzing the germline precursors of broadly neutralizing antibodies, immunogens can be engineered to preferentially activate naïve B cells capable of evolving into protective antibody lineages.³⁹¹⁻³⁹⁴ In parallel, machine learning and artificial intelligence approaches are increasingly being applied to predict antigen–antibody interactions and optimize immunogen design.³⁹⁵⁻³⁹⁷ These technologies may accelerate the development of next-generation vaccines capable of eliciting broadly protective responses against multiple orthoflaviviruses.

Collectively, the findings presented here contribute to a growing understanding of how antibody responses target structural vulnerabilities at the orthoflavivirus EDIII protein. The identification of antibodies capable of recognizing conserved epitopes across distinct viruses, combined with structural insights into their mechanisms of neutralization, provides a conceptual framework for the development of both therapeutic antibodies and rationally designed vaccines aimed at combating emerging orthoflavivirus threats.

Future Directions

Several avenues of investigation emerge from the findings described in this work.

First, broader surveys of human antibody repertoires following orthoflavivirus infection could help clarify the extent to which cross-reactive EDIII antibodies arise after sequential orthoflavivirus exposure. High-throughput B-cell sequencing combined with mAb isolation and characterization are expected to provide insight into the evolutionary pathways that give rise to broadly reactive antibodies.

Second, additional ecological and virological studies are needed to determine whether undiscovered orthoflaviviruses circulate in regions where cross-reactive antibodies have been detected. Metagenomic sequencing of arthropod vectors and wildlife reservoirs may reveal previously unrecognized viruses that contribute to shaping human immune responses.

Finally, advances in computational protein design offer powerful tools for the rational engineering of vaccine immunogens. By integrating structural biology, antibody repertoire analysis, and machine learning approaches, it may become possible to design vaccines that present conserved neutralizing and protective epitopes while avoiding immunodominant but non-protective regions. Such strategies could lead to the development of broadly protective vaccines against multiple orthoflaviviruses, addressing a major and still only partially met need in global infectious disease control.

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