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THE HUMAN ANTIBODY RESPONSE TO TICK-BORNE FLAVIVIRUSES

Dissertation

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“The most exciting phrase to hear in science, the one that heralds the most discoveries, is not ‘Eureka!’ but 'That's funny…'”

Isaac Asimov (1920–1992)

SUMMARY

Flaviviruses are well known to be a world-wide threat and the lack of vaccines and treatments for most of them indicates the need to reduce the current impact of flaviviruses on public health. The flavivirus genus includes viruses such as dengue (DENV), Zika (ZIKV) and West-Nile (WNV) that are spread by mosquitoes and cause human disease and mortality in tropical areas. In contrast, Powassan (POWV) and tick-borne encephalitis viruses (TBEV) can cause severe neuroinvasive disease and mortality in temperate regions of the Northern hemisphere that are populated by their tick vectors. Flaviviruses are characterized by virus-specific as well as cross-reactive immune responses. The latter could potentially be harnessed for the design of broadly effective vaccines and other therapies but might also promote adverse immune responses such as through antibody-dependent enhancement (ADE) of infection and disease.

The overall goal of this thesis was a better understanding of the characteristics of the human immune response to flaviviruses by (I) characterizing the cross-reactivity of human anti-ZIKV antibodies to tick-borne flaviviruses (TBV) and by (II) identifying individuals exposed to POWV and studying their antibody response to POWV. In addition, a secondary aim was to expand the library of available reporter flavivirus particles (RVP) for future neutralization and cross-neutralization studies.

(I) Out of a panel of 42 human anti-ZIKV antibodies we found two, Z012 and Z021, that strongly bound to TBEV and POWV, respectively. Interestingly, Z021, which is a potent neutralizer of ZIKV and a promising therapeutic agent for preventing ZIKV fetal disease, showed poor neutralization and also ADE of POWV infection *in vitro*. Cross-reactivity of anti-ZIKV antibodies to TBV has not been described previously, thus this finding could lead to the discovery of a novel epitope that is shared between ZIKV and POWV and improve our understanding of the cross-reactivity between mosquito-borne and tick-borne flaviviruses.

(II) To identify POWV exposed individuals we screened 591 samples from New York residents exposed to ticks because of confirmed or suspected Lyme disease. As control, we included in the analysis 49 samples from Brazil following a Dengue outbreak in 2010 and 109 samples from Mexico obtained in a dengue endemic region following a Zika outbreak in 2016. Unexpectedly, we discovered remarkable neutralizing activity against POWV in samples from individuals living in tropical Mexico where POWV has not been described before. Furthermore, using single-cell methods, we discovered human antibodies against POWV, out of which two not only recognized and neutralized POWV but also cross-reacted to ZIKV, WNV and Usutu virus. Experiments are ongoing to characterize this antibody response and to determine whether the neutralizing activity results from exposure to POWV or to a novel POWV-like virus in tropical Mexico or from cross-reactive antibodies elicited by the mosquito-borne DENV and ZIKV that are endemic in that region.

Altogether, this research bears the potential to discover antibodies that can be developed for diagnostic tests, prevention, postexposure prophylaxis and treatment of patients with POWV disease. Human anti-POWV monoclonal antibodies can furthermore guide the development of POWV vaccines through the identification of neutralizing epitopes.

ZUSAMMENFASSUNG

Flaviviren sind als weltweite Bedrohung bekannt, und der Mangel an Impfstoffen und Therapien für die meisten Flaviviren zeigt, dass der derzeitige Einfluss von Flaviviren auf die öffentliche Gesundheit verringert werden muss. Die Gattung der Flaviviren umfasst Viren wie Dengue (DENV), Zika (ZIKV) und West-Nil (WNV), die von Mücken übertragen werden und in tropischen Gebieten Krankheiten und Sterblichkeit beim Menschen verursachen. Im Gegensatz dazu können Powassan- (POWV) und der Frühsommer-Meningoenzephalitis-Virus (TBEV) in gemäßigten Regionen der nördlichen Hemisphäre, die von ihren Zeckenvektoren besiedelt sind, schwere neuroinvasive Erkrankungen und Mortalität verursachen. Flaviviren sind durch virusspezifische sowie kreuzreaktive Immunantworten gekennzeichnet. Letztere könnten potenziell für die Entwicklung breit wirksamer Impfstoffe und anderer Therapien genutzt werden, aber sie könnten auch nachteilige Immunantworten fördern, wie beispielsweise durch eine antikörperabhängige Verstärkung (ADE) von Infektionen und Krankheiten.
Das übergeordnete Ziel der Arbeit war ein besseres Verständnis der Eigenschaften der menschlichen Immunantwort auf Flaviviren durch (I) Charakterisierung der Kreuzreaktivität menschlicher Anti-ZIKV-Antikörper gegen durch Zecken übertragene Flaviviren (TBV) und durch (II) Identifizierung von POWV-exponierten Personen sowie die Untersuchung ihrer Antikörperantwort auf POWV. Ein sekundäres Ziel war es außerdem, die Bibliothek der verfügbaren Reporter-Flavivirus-Partikel (RVP) für zukünftige Neutralisations- und Kreuzneutralisationsstudien zu erweitern.
(I) Aus einer Gruppe von 42 humanen Anti-ZIKV-Antikörpern fanden wir zwei, Z012 und Z021, die stark an TBEV bzw. POWV gebunden haben. Interessanterweise zeigte Z021, ein starker Neutralisator von ZIKV und ein vielversprechendes therapeutisches Mittel zur Vorbeugung von fetalen Erkrankungen durch ZIKV, eine schlechte Neutralisation und auch eine ADE der POWV-Infektion *in vitro*. Die Kreuzreaktivität von Anti-ZIKV-Antikörpern gegen TBV wurde bisher nicht beschrieben. Daher könnte dieser Befund zur Entdeckung eines neuen Epitops führen, das ZIKV und POWV gemeinsam haben, und unser Verständnis der Kreuzreaktivität zwischen von Mücken und von Zecken übertragenen Flaviviren verbessern.
(II) Um POWV-exponierte Personen zu identifizieren, untersuchten wir 591 Proben von New Yorker Bewohnern, die aufgrund einer bestätigten oder vermuteten Lyme-Borreliose Zecken ausgesetzt waren. Als Kontrolle bezogen wir 49 Proben aus Brasilien nach einem Dengue-Ausbruch im Jahr 2010 und 109 Proben aus Mexiko aus einer Dengue-endemischen Region nach einem Zika-Ausbruch im Jahr 2016 in die Analyse ein. Unerwarteterweise entdeckten wir in Proben von im tropischen Mexiko lebenden Personen, wo POWV bisher noch nicht beschrieben wurde, eine bemerkenswerte neutralisierende Aktivität gegen POWV. Darüber hinaus entdeckten wir mithilfe von Einzelzell-Methoden humane Antikörper gegen POWV, von denen zwei nicht nur POWV erkannten und neutralisierten, sondern auch mit ZIKV, WNV und Usutu-Virus kreuzreagierten. Es werden Experimente durchgeführt, um diese Antikörperantwort zu charakterisieren und um festzustellen, ob die neutralisierende Aktivität aus der Exposition gegenüber POWV oder einem neuartigen POWV-ähnlichen Virus im tropischen Mexiko

resultiert oder durch kreuzreaktive Antikörper verursacht wird. Letztere könnte durch die von Mücken übertragenen DENV und ZIKV hervorgerufen werden, die in dieser Region endemisch sind.
Insgesamt birgt diese Forschung das Potenzial, Antikörper zu entdecken, die für diagnostische Tests, Prävention und Postexpositionsprophylaxe sowie für die Behandlung von Patienten mit der POWV Erkrankung entwickelt werden können. Humane monoklonale Anti-POWV-Antikörper können darüber hinaus die Entwicklung von POWV-Impfstoffen durch die Identifizierung neutralisierender Epitope steuern.

LIST OF ABBREVIATIONS

%	percent
°C	degree celsius
ADE	Antibody dependent enhancement
ATCC	American Type Culture Collection
BSA	Bovine Serum Albumin
BT50	50% binding titer
C protein	Capsid protein
CDC	Centers for Disease Control and Prevention
CIP	Calf Intestinal Phosphatase
CNS	central nervous system
COVID-19	coronavirus disease-2019
CprME	flaviviral structural proteins
CSF	cerebrospinal fluid
DENV	Dengue virus
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	desoxyribonucleic acid
dNTP	desoxynucleoside triphosphate
E protein	envelope protein
EC50	half maximal effective concentration
EDI/II/III	envelope domain I/II/III
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
Env	Human Immunodeficiency Virus envelope glycoprotein
Fab	antigen-binding fragment
FBS	Fetal Bovine Serum
FcγR	Fcγ receptors
g	gramm
GRLR	Fc mutated version that prevents FcγR binding
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
HIV	Human Immunodeficiency Virus
hr	hour
HRP	horseradish peroxidase
HSV	Herpes Simplex Virus
IC50	half maximal inhibitory concentration
IDT	Integrated DNA Technologies
i.e.	id est
IFA	immunofluorescence assay

Ig	immunoglobulin
JEV	Japanese Encephalitis Virus
Kb	kilobase
KFDV	Kyasanur Forest disease virus
l	liter
LGTV	Langat Virus
LIV	Louping ill Virus
m	milli, 10^{-3}
µ	micro, 10^{-6}
M	Molar
M protein	membrane protein
MBV	mosquito-borne flaviviruses
MIA	microsphere-based immunoassay
min	minute
mRNA	messenger RNA
MVEV	Murray Valley encephalitis virus
n	nano, 10^{-9}
NKV	No Known Vector
NT50	50% neutralization titer
OD	Optical Density
OHFV	Omsk hemorrhagic fever Virus
PBMC	peripheral blood mononuclear cells
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline with 0.05% Tween 20
PCR	polymerase chain reaction
POWV	Powassan Virus
POWV-DTV	Powassan Virus, Deer Tick Virus-strain
POWV-LB	Powassan Virus, LB-strain
prM protein	precursor of membrane protein
PRNT	plaque reduction neutralization test
RLU	Relative Luciferase Units
RNA	ribonucleic acid
rpm	rounds per minute
RT-PCR	Real-time polymerase chain reaction
RVPs	Reporter Viral Particles
SA-APC	Streptavidin-Allophycocyanin
SA-PE	Streptavidin-Phycoerythrin
SARS-CoV-2	severe acute respiratory syndrome-related coronavirus-2
SD	standard deviation
sec	second

SLEV	St. Louis encephalitis virus
TAE	Tris-Acetate-EDTA
TBEV	Tick borne encephalitis Virus
TBEV-EU	Tick borne encephalitis Virus, European subtype
TBEV-FE	Tick borne encephalitis Virus, Far Eastern subtype
TBEV-SI	Tick borne encephalitis Virus, Siberian subtype
TBV	tick-borne flaviviruses
TMB	3,3',5,5'-Tetramethylbenzidine
USUV	Usutu Virus
V	Volt
WHO	World Health Organization
WNV	West Nile Virus
xg	times gravity
YFV	Yellow Fever Virus
ZEDIII	Zika Virus Envelope Domain III
ZIKV	Zika Virus

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1 INTRODUCTION

1.1 FLAVIVIRUSES

The Flavivirus genus is well known to be a world-wide threat to public health: The global spread of dengue virus (DENV) with more than 390 millions of estimated human infections yearly, explosive Zika virus (ZIKV) outbreaks first across the Pacific and then in the Americas since 2013 and the inherent danger of yellow fever virus (YFV) epidemics in Africa and South America are examples for the immense effect of flaviviruses on global health (1).
The flavivirus genus includes over 50 arthropod-borne viruses, some of which can cause a broad range of disease in humans including hemorrhage, meningitis and encephalitis as well as death (2).
In total, effective vaccines have been developed against only 5 flaviviruses (DENV, YFV, Japanese Encephalitis Virus (JEV), Tick borne encephalitis virus (TBEV), Kyasanur Forest disease virus (KFDV)) (2). Furthermore, no specific treatments are available to treat flavivirus infections (2).

1.1.1 FLAVIVIRUS GENOME AND STRUCTURE

Flaviviruses are spherical in shape and share a common enveloped structure, approximately 50nm in diameter, and include a positive, single-stranded 11kb RNA genome (2).
The genome is translated into one polyprotein that is cleaved into 7 non-structural proteins and 3 structural proteins (2). The non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) are encoded at the 3' end of the genome and contribute to viral replication, to the processing of the large polyprotein as well as to the evasion of the cellular antiviral immune response (3). The 5' end encodes the structural proteins: C, prM/M, E. The capsid (C) protein interacts with the genomic RNA and forms the nucleocapsid complex (3). The precursor of membrane (prM) protein stabilizes the immature virion by preventing fusion with host cell membranes (4). The pr peptide is released by cleavage of the prM protein, when budding from the host, and the membrane (M) protein stays anchored in the membrane of the mature virus (4). The envelope (E) protein mediates virus attachment to cellular receptors and membrane fusion in endosomes after the virus was taken up by endocytosis (1).
The flaviviral surface consists of a cell derived lipid membrane, in which 180 viral E proteins and M proteins are embedded with their transmembrane domains (3). The E proteins form antiparallel homodimers in a herringbone pattern, whereas 3 dimers build one raft (Fig. 1). However, the flavivirus particle is not a static structure but shows flexible and dynamic conformational changes, referred to as "viral breathing", which can affect the virus neutralizing potency of antibodies (5).

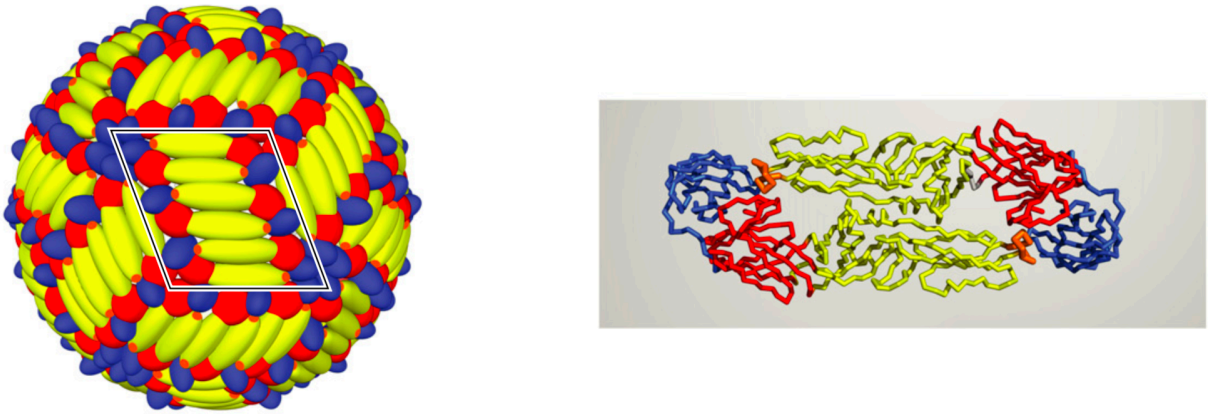

Fig. 1: Diagram of the surface of the mature flavivirus particle and E homodimer (from Rey et al., 2017).
The E protein is colored according to domains: red, yellow and blue for domains I, II and III, respectively. The fusion loop is highlighted in orange. ***Left****: Surface of a mature flavivirus particle, consisting of 90 E protein homodimers forming a herringbone pattern. One raft of three E homodimers is framed in black.* ***Right****: Top view of a single E homodimer.*

1.1.2 Antibodies against Flaviviruses and cross-reactivity

Because flavivirus E proteins have a highly conserved structural organization, antibodies that develop against one flavivirus can also recognize others, and this cross-reactivity can lead to cross-protection (6, 7). In this context, the degree of amino acid identity of the E protein strongly correlates with the extent of cross-neutralization (6). Serum cross-neutralization between flaviviruses has led to the subdivision into serocomplexes such as the dengue, the Japanese encephalitis and the tick-borne encephalitis serocomplexes (8). Cross-neutralization by polyclonal sera between different serocomplexes is usually not observed (8), and, thereby, it is assumed that cross-neutralization by polyclonal sera is lost when the amino-acid divergence of the E protein is higher than 50% (6). However, a few studies have shown cross-reactive immune responses among flaviviruses from differing serocomplexes (9, 10).

1.1.3 Mosquito-borne and tick-borne Flaviviruses

Flaviviruses can be divided into three main groups, depending on the vector by which they are transmitted to vertebrates: the tick-borne flaviviruses (TBV), the mosquito-borne flaviviruses (MBV) and those with No Known Vector (NKV) (Fig. 2).

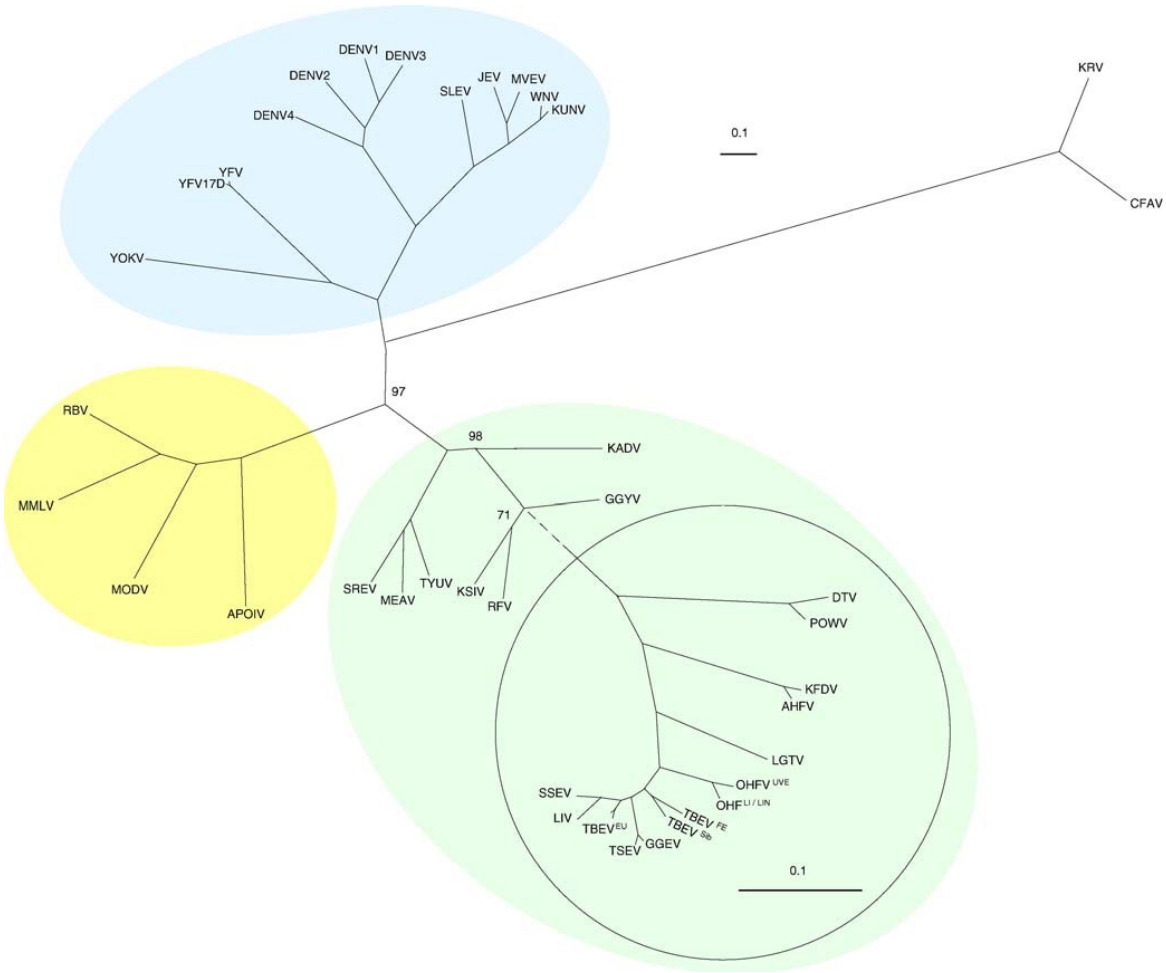

Fig. 2: Phylogenetic analysis based on complete polyprotein sequences (from Grard et al., 2007). *"Phylogenetic reconstruction was performed using the maximum likelihood method. All branchings were supported by quartet puzzling frequencies at 99% or 100% except at the forks where a value is indicated. The tick-borne flavivirus group is highlighted in green, the mosquito-borne flavivirus group in blue and the no-known vector flavivirus group in yellow. To improve the legibility of the tree, the distal part of the TBV branch is presented with a 3.5× magnification."(11)*

Mosquito-borne flaviviruses (MBV)

MBV are rapidly evolving and can travel over long distances by infected people or animals, especially migratory birds, as well as within mosquito eggs (12). MBV epidemics happen often after periods of high temperature and humidity, which give rise to mosquitoes, and are ended by cooler weather (12). Mosquitoes such as those of the *Aedes* and *Culex* species serve as vectors for a variety of flaviviruses: *Aedes* flaviviruses are often associated with hemorrhagic disease syndromes (see for example YFV and DENV), whereas *Culex* Flaviviruses include viruses such as West Nile Virus (WNV) and JEV that cause encephalitic disease syndromes (12).

Dengue viruses (DENV)

40% of the world's population, about 3 billion people, live in areas with a risk of dengue infection (13). DENV consists of 4 closely related but antigenically different serotypes (DENV1, DENV2, DENV3, DENV4) that are all pathogenic in humans and potentially cause dengue hemorrhagic fever and, less commonly, dengue shock

syndrome (2). A DENV vaccine is approved in some countries for specific populations with confirmed prior dengue infection (14).

Zika virus (ZIKV)

ZIKV was a neglected MBV with infrequent reports of mild febrile disease in Africa and Asia until it caused massive outbreaks across the Pacific and in the Americas since 2013 (6). ZIKV can cause severe neurological complications such as meningoencephalitis and Guillain-Barré-Syndrome (15). Infection during pregnancy is linked to fetal aberrations, including microcephaly, that are referred to as congenital Zika syndrome (15).

Yellow Fever virus (YFV)

YFV circulates in tropical and subtropical areas of Africa and South America and causes hemorrhagic fever with jaundice in people who are severely affected (2). A safe and effective yellow fever vaccine has been available for more than 80 years and has restrained YFV emergence in many regions (2). However, outbreaks in Angola, Congo and Brazil still occur, demonstrating that large populations with low vaccination rates are in danger of re-emerging urban YFV epidemics.

Japanese encephalitis virus (JEV)

JEV leads to serious neuroinvasive disease often resulting in persistent neurological damage (2). It is the leading cause of vaccine-preventable encephalitis in Asia and the Western Pacific causing up to 50,000 cases of encephalitis every year with case fatality rates of about 25% (12).

Murray valley encephalitis virus (MVEV)

MVEV is a member of the Japanese encephalitis serocomplex and is endemic to northern Australia and Papua New Guinea (16). It can lead to fatal encephalitis in horses and is the most important cause of human arboviral encephalitis in Australia (16).

West Nile virus (WNV)

The occurrence of WNV infection is broadly distributed world-wide and can result in neuroinvasive disease in less than 1% of infections (2). The neuroinvasive disease can be lethal and cause symptoms that persist beyond a year from infection (2). An unexpected outbreak of fever and encephalitis in New York in 1999, shortly after its introduction in the Americas, marked the emergence of WNV as a major threat (12).

St. Louis encephalitis virus (SLEV)

SLEV has only been isolated in the Americas (12). It is related to JEV and WNV and can also cause severe neuroinvasive disease, more commonly in older adults, with rare cases of long-term disability and fatality rates of about 7% (12).

Usutu virus (USUV)

USUV is closely related to WNV and was first identified in South Africa in 1959 (17). In Europe, it was first reported in Austria in 2001 following a significant die-off of birds,

after which researchers observed the introduction to neighboring European countries (17). Five human cases of USUV-related neuroinvasive illness were reported in Italy and Croatia. USUV has also been associated with clinical diagnosis of idiopathic facial paralysis (17).

Tick-borne flaviviruses (TBV)

TBV are transmitted by a variety of ticks and are important livestock and human pathogens (2). In humans, TBV can cause encephalitis or hemorrhagic fever, whereas lesser-known members are not associated with human or animal disease (3). TBV with medical importance are globally distributed over the Northern Hemisphere (3). They are assumed to have undergone gradual evolution due to several factors: ticks can live up to five years, whereas they feed only three times (as larvae, nymphs and adults) and co-feeding on rodents in the forests can lead to transmission of viruses between ticks (12). These factors indicate that replication in vertebrate hosts is not necessary for TBV to survive over a long period of time (12).

Tick borne encephalitis virus (TBEV)

With an estimated 10,000 to 15,000 cases per year in Europe and Asia, TBEV is the most significant human TBV (3). There are three subtypes of TBEV: The Western European (TBEV-EU), the Siberian (TBEV-SI) and the Far Eastern (TBEV-FE) subtype. All three subtypes can cause encephalitis and meningitis whereas the case fatality rate ranges between 1% and 20% for TBEV-EU and TBEV-FE, respectively (3). Four vaccines for TBEV are available, which have significantly reduced the incidence of TBEV in endemic areas (3).

Louping ill virus (LIV)

LIV is a descendant of TBEV and the only TBV found in the British Isles (18).
LIV causes a febrile illness in sheep, cattle and some other species, that can progress to fatal encephalitis (18). Since 1934, while 45 cases of human infections have been reported, mainly after occupational exposure to infected livestock, only one human died as a consequence of the infection (18).

Kyasanur forest disease virus (KFDV)

KFDV was identified in 1957 when it was isolated from sick and dying monkeys from the Kyasanur Forest in India (12). Since then, between 100 to 500 human cases per year have been reported with a case fatality rate of 2 to 10% (12). KFDV can lead to febrile illness with severe hemorrhagic or neurological symptoms (19). A vaccine for KFDV does exist and it is used in endemic areas of India since 1990 (19).

Omsk hemorrhagic fever virus (OHFV)

OHFV was firstly isolated in Omsk, west Siberia, in 1947 and can cause severe and potentially lethal human disease (20). Human disease includes hemorrhagic fever with similar symptoms as caused by KFDV and is associated with outbreaks in muskrats that infect humans (20).

Langat virus (LGTV)

LGTV is a member of the TBEV serocomplex, however there are no known cases of human diseases associated with it (21). It was studied extensively in clinical trials to develop a live TBEV vaccine, because it was found to induce a protective, durable immune response (21). Although the LGTV-based vaccine was more effective in preventing TBEV in endemic regions than other vaccines at that time, further clinical studies were stopped because of neurological disease in 1 of every 20,000 vaccinations (21).

1.2 POWASSAN VIRUS (POWV)

A less studied member of the TBV is Powassan virus (POWV). POWV is the only TBV known to be present in North America. It can cause a rare but severe and fatal neuroinvasive disease. POWV was first identified in 1958 after isolation from the brain of a 5 year old boy from Powassan, Ontario, who succumbed to encephalitic disease (22). The recent rising incidence of human POWV disease is concerning given the potential severity of disease and the absence of specific treatment options.

As of today, we know of two lineages of POWV: The LB strain (POWV-LB), that was isolated from the first fatal case in Powassan, and the Deer Tick Virus (POWV-DTV) (23-25). POWV-DTV was initially assumed to cause only mild or asymptomatic disease and be distinct from POWV-LB (26, 27), but this was refuted in subsequent studies. Both lineages share 94% amino acid identity (25), are clinically and serologically indistinguishable (23, 25) and have been linked to fatal human disease (22, 28).

1.2.1 EPIDEMIOLOGY

Fig. 3: Geographical distribution of POWV symptomatic individuals (from Kemenesi and Bányai, Clinical Microbiology Reviews 2019).
Red dots indicate where POWV was isolated and/or serologically identified.

POWV occurs in wide regions of the Northern Hemisphere including North America and Russia (3). It is the only member of the encephalitogenic TBV that is known to be present in North America (29). Most of the US cases occur in the North-central and -eastern states such as Massachusetts, Minnesota, New York and Wisconsin (3). In Canada, human POWV cases have been mainly described in the east such as in New Brunswick, Quebec and Ontario (3). In Russia, POWV disease has been identified in the far eastern Primorski province (30). Early serology data from 1962 indicated that POWV may occur in the northwest Senora state of Mexico (31). However, this has not been described in subsequent studies.
While POWV disease is rare, the incidence seems to be increasing since the late 1990s (30): Between 1958 and 1998 only 27 human cases of POWV disease were described in North America (32). In contrast, from 2001 to 2019, 186 human POWV disease cases were reported only in the United States, and the yearly number of reported cases is rising (Fig. 4). In 2019, a record number of 39 cases were reported to the Centers for Disease Control and Prevention (CDC). Many factors may have contributed to the recent rise of POWV disease incidence such as increased surveillance and diagnosis as well as the recent geographic expansion of an important tick vector, the *Ixodes scapularis* (33).

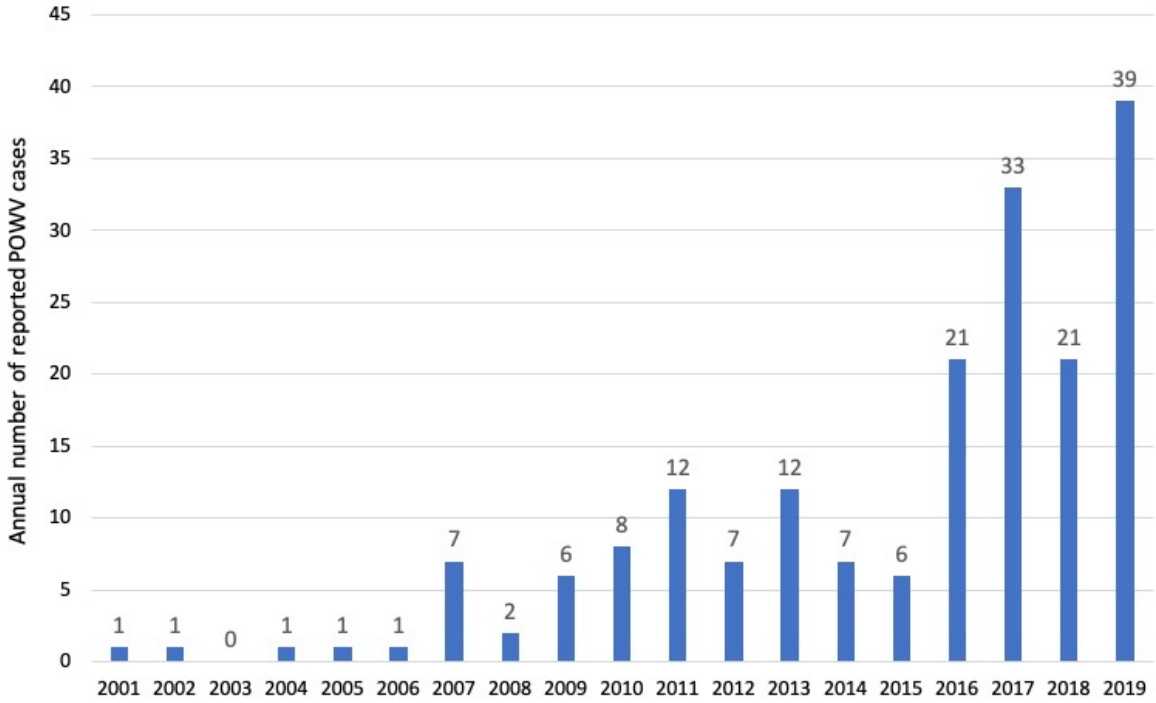

Fig. 4: Annual number of POWV cases in the United States reported to the Centers for Disease Control and Prevention from 2001 to 2019 (34, 35).

In North America, seroprevalence of POWV in the general population differs from state to state. Studies describe seroprevalence rates in Ontario, British Columbia, New York, Minnesota and Wisconsin ranging from 0.7% to 6.1% (3). The high seroprevalence rates compared to the low incidence of human disease suggest that either most POWV infections are asymptomatic or mild or that tests are not sufficiently specific so that POWV infections remain undetected. Also, coinfections with other tick-borne pathogens could mask symptomatic POWV infections (36).

1.2.2 POWV TRANSMISSION

POWV infections are tick-borne and mainly occur from May to November, when ticks are most active (37). The most important vectors of POWV in North America are ticks belonging to the *Ixodes* species (3). POWV detection rates in *Ixodes* ticks have been reported at around 1% to 6.5% (38-43).
The two lineages of POWV are associated with distinct vectors feeding upon different vertebrate hosts (30). POWV-LB is linked to two enzootic cycles: The first cycle includes *Ixodes cookei* feeding upon groundhogs as well as skunks. The second cycle consists of *Ixodes marxi* feeding upon squirrels (30). In contrast, POWV-DTV is maintained by *Ixodes scapularis* and a variety of vertebrate hosts such as deer and white-footed mice (3).
In the natural enzootic cycle humans are considered dead-end hosts for POWV who can be infected by all three *Ixodes* species (30). However, the public health risk is assumed to be higher for *Ixodes scapularis* than for the other two species because of its low host-specificity and aggressive feeding behavior (29). In addition, *Ixodes scapularis* is drastically expanding its territory: As recently reviewed, the number of counties in the United States in which *Ixodes scapularis* has been established has more

than doubled over the past two decades (33). In accordance with this finding, in Connecticut the prevalence of antibodies to POWV-DTV in deer, upon which *Ixodes scapularis* feed, has dramatically increased from 4% in 1979 to 91% in 2009 (44).
The tick attachment time for effective transmission of POWV-DTV to mice was described to be as little as 15 min (45). However, the exact time for effective tick-to-human transmission of POWV is unknown. In one human POWV case a relatively short tick attachment time of less than 3hr was linked to severe POWV neuroinvasive disease (46). In contrast, for other non-viral tickborne pathogens such as Borrelia burgdorferi the risk of transmission is assumed to be minimal if the tick is removed within 24hr (29). Therefore, removing ticks from the skin within 24hr of attachment may prevent the transmission of other non-viral tickborne pathogens whereas POWV may be still transmitted.

1.2.3 POWV CLINICAL PRESENTATION

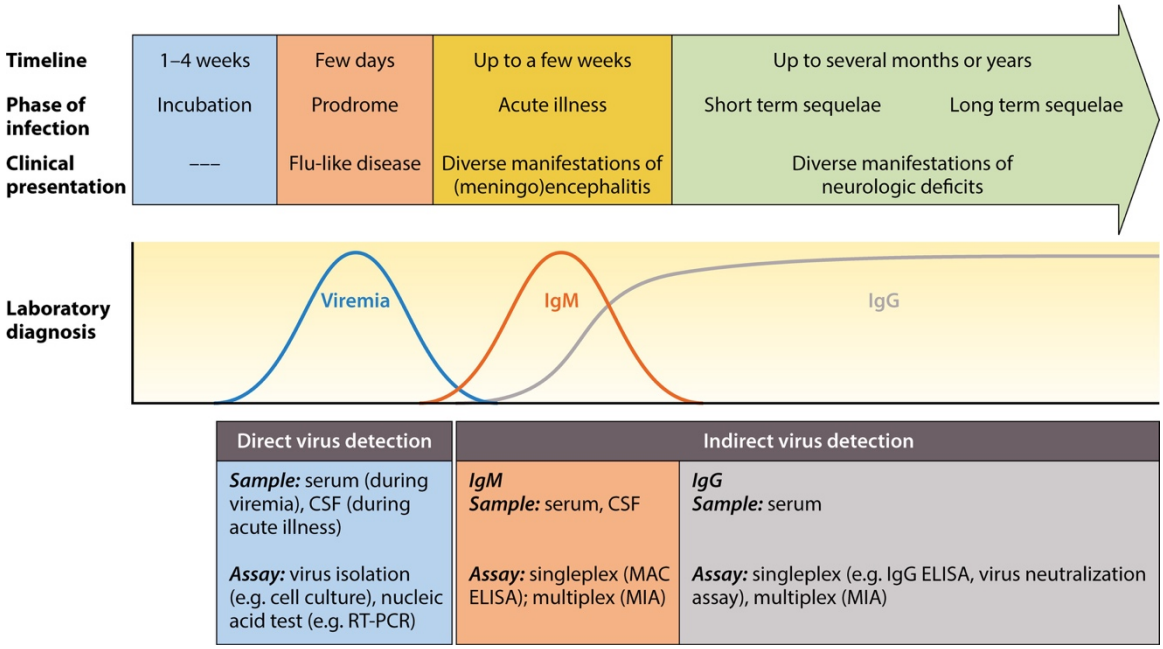

Fig. 5: Clinical presentation and diagnostic laboratory tests over the course of POWV infections (from Kemenesi and Banyal, 2019)

Symptomatic POWV infections can be divided into 4 phases (Fig. 5). Data from 44 patients from documented case reports, case series analyses and review articles have been comprehensively studied in a recent review (3), which outlined the following clinical presentation of each phase:
The incubation period ranges from about 1 week to 1 month, in which patients are asymptomatic. This period is followed by a 1 to 3 day prodrome phase manifesting in a flu-like disease. Symptoms can include fever, chills, headache, malaise and a sore throat. After the nonspecific flu-like illness, patients develop encephalitis and/or meningitis that can last up to a few weeks. This acute illness is characterized by diverse central nervous system (CNS) symptoms: The spectrum of symptoms can range from mild (fever, headache, dizziness, drowsiness, neck stiffness) to severe (pyramidal tract signs, quadriplegia, coma, respiratory failure).
Approximately 75% of reported survivors suffer from diverse short- or long-term

neurological sequelae. Short-term sequelae are apnea, psychosis, drowsiness, altered sensorium, dysarthria or mutism and spasticity or rigidity. Long-term sequelae can manifest as prolonged headache, depression, memory dysfunction, quadriplegia, aphasia, mental retardation and bed bound state as well as dependence on ventilator. Approximately 10 to 15% of POWV neuroinvasive disease cases are fatal (3).

1.2.4 POWV DIAGNOSIS

POWV infections can be diagnosed by either direct or indirect virus detection (Fig. 5). Direct virus detection is possible before seroconversion during the initial phase of infection. Serum from the viremic phase and cerebrospinal fluid (CSF) during the acute illness can be used to detect POWV RNA by reverse transcription-PCR (RT-PCR) (3). However, serologic diagnostics for POWV are more relevant during the encephalitic phase when patients have to be hospitalized and POWV is cleared from the blood and CSF. Therefore, the indirect virus detection by serology including usage of the enzyme-linked immunosorbent assay (ELISA), immunofluorescence assay (IFA) and microsphere-based immunoassay (MIA) is considered best practice (3). Such assays are presumptive and should be confirmed by for example a plaque reduction neutralization test (PRNT) to detect POWV specific neutralizing antibodies (47). According to the CDC, a case of POWV neuroinvasive illness is confirmed when it meets the clinical criteria (any signs of central or peripheral neurologic dysfunction and the absence of a more likely clinical explanation) and at least one of the following laboratory criteria:

- POWV isolation from, or demonstration of specific viral antigen or nucleic acid in, tissue, blood, CSF, or other body fluid, OR
- ≥ 4-fold change in POWV-specific quantitative antibody titers in paired sera, OR
- POWV-specific immunoglobulin M (IgM) antibodies in serum with confirmatory POWV-specific neutralizing antibodies in the same or a later specimen, OR
- POWV-specific IgM antibodies in CSF, with or without a reported pleocytosis, and a negative result for other IgM antibodies in CSF for arboviruses endemic to the region where exposure occurred (47).

When interpreting laboratory results, it is important to consider that flaviviruses can produce cross-reactive antibodies. Thus, the patient's vaccination and travel history should be taken into account. A complex serology for more than one flavivirus may be needed when two or more closely related arboviruses occur in the same region of the patient's exposure (47).

1.2.5 POWV DIFFERENTIAL DIAGNOSIS

A broad differential diagnosis is indispensable because neuroinvasive disease can be caused by many different viruses and bacteria that occur in the same or overlapping regions as POWV such as TBEV, WNV, SLEV, herpes simplex virus (HSV); bacterial meningitis; Lyme disease; anaplasmosis; ehrlichiosis; and tick-borne relapsing fever (3). In addition, vector surveillance data from Connecticut and New York show that *Ixodes scapularis* ticks infected with POWV-DTV are coinfected with other pathogens (*Borrelia burgdorferi, Ehrlichia chafeensis, Anaplasma phagocytophilum* or a combination thereof) at a detection rate of 1.1% to 3.4% (3, 48, 49). Supporting these findings,

studies report tick-borne pathogen coinfections with *Anaplasma phagocytophilum* and *Borrelia burgdorferi* in patients with POWV infection (50-52). These coinfections can complicate diagnostics and treatment of patients with POWV illness and increase morbidity and mortality (51).

1.2.6 POWV PREVENTION AND TREATMENT

Several reports describe the treatment of POWV neuroinvasive disease with high-dose corticosteroids (52-54), intravenous Ig (55) and antiviral therapy with pegylated interferon alpha and ribavirin (56). However, the effect of these treatments on POWV disease outcome remains unclear (29). Currently, no specific treatments or vaccines for POWV disease are available. Thus, reducing exposure to ticks is the best available prevention of infection (29). For severe POWV illness, symptomatic treatment may include hospitalization with respiratory support, intravenous fluids and medication to reduce cerebral edema (29).

1.3 ANTIBODIES TO FLAVIVIRUSES

1.3.1 ANTIBODY MEDIATED NEUTRALIZATION OF FLAVIVIRUSES

Antibodies are sufficient for neutralizing flaviviruses and protecting the host from infection, as demonstrated by serum and antibody transfer experiments in mice (57, 58). Additionally, potently neutralizing monoclonal antibodies were shown to be protective in several animal models of ZIKV and DENV infection when administered before or a short period after infection (6, 59, 60).
It is estimated that at least 30 antibody molecules per virion are required for neutralization, whereas antibody affinity and epitope accessibility are critical factors that determine whether neutralization occurs (57, 61).
The E protein is responsible for cell entry and is, in consequence the main target of neutralizing antibodies (62). It contains three structural domains (EDI, EDII and EDIII) that are connected by flexible linkers (63, 64): EDII is a long finger-like domain that contributes to the dimerization of E proteins and mediates the membrane fusion after virus uptake by a highly conserved fusion loop at its distal end (5). EDIII has an Ig-like structure that includes receptor binding sites, whereas EDI connects EDII and EDIII (65).
Antibody responses to the E protein differ in neutralizing capacity and cross-reactivity with related flaviviruses depending on what domain the antibodies target. The humoral response against flaviviruses consists of a large fraction of antibodies against the EDII (66). However, these antibodies are mostly cross-reactive and less potent (5, 66). In contrast, antibodies to the EDIII are generally more flavivirus-specific and among the most potent neutralizers (67-69).

1.3.2 ANTIBODY DEPENDENT ENHANCEMENT (ADE) OF INFECTION

Cross-reactivity of antibodies against multiple flaviviruses is a characteristic that could potentially be harnessed for the design of broadly acting vaccines and other therapies. However, there is concern that cross-reactive antibodies that are non- or only poorly neutralizing could actually enhance infection, by a phenomenon referred to as antibody dependent enhancement (ADE) (70). Much of the understanding about ADE is based on DENV studies that associate pre-existing DENV antibody titers with severe forms of DENV disease (dengue hemorrhagic fever and dengue shock syndrome) typically during secondary infections with a distinct DENV serotype (71, 72). Evidence for ADE exists not only for flaviviruses but also for other viruses of different families (70).

At least two mechanisms have been proposed to explain ADE: extrinsic ADE (increased virus uptake) and intrinsic ADE (suppression of innate antiviral cell responses) (6). In the extrinsic form of ADE, the virus is bound by non-neutralizing antibodies or antibodies at sub-neutralizing concentrations. These virus-antibody complexes are internalized by Fcγ receptors (FcγR) mediated endocytosis leading to enhanced infection of the phagocytic cell (6). Extrinsic ADE requires pre-existing antibodies due to a prior sensitization of the humoral immune response (70).
In the intrinsic form of ADE, the internalized immunocomplexes modulate the innate

antiviral cell responses by FcγR mediated signaling which leads to increased virus production in the cell (6).
Both, the extrinsic and intrinsic ADE mechanisms are thought to contribute to increased disease severity by enhancing virus production and causing massive release of inflammatory and vasoactive mediators (70).
ADE is a major obstacle to vaccine development for many viruses (70). In the case of Dengvaxia®, a vaccine against DENV, in 2017 the manufacturer announced that people who were not infected with DENV prior to vaccination may be at risk of developing severe dengue if they are re-infected by DENV later on (14). As a consequence, the World Health Organization (WHO) now recommends that Dengvaxia® should only be used on individuals with confirmed prior DENV exposure (14).

1.3.3 Methods for the molecular characterization of anti-flavivirus antibodies

In 2009 Michel Nussenzweig's laboratory at the Rockefeller University, which I joined for this thesis, established single-cell antibody cloning methods to discover human monoclonal antibodies against human immunodeficiency virus 1 (HIV-1) (73). With this technique, B cells were isolated based on their ability to bind to the HIV-1 envelope glycoprotein (Env) and their RNA was extracted to clone anti-HIV-1 antibodies (73, 74). This method led to the identification of potent antibodies targeting different sites on the HIV-1 Env. Further, structural and biochemical analysis enabled a detailed molecular understanding of neutralizing epitopes that are now instructing the design of candidate vaccines (75). Hundreds of new antibodies to HIV-1 were cloned that were 2 or 3 orders of magnitude more potent in virus neutralization assays than those available before 2009 (75). Some of these antibodies are currently in phase 2 clinical trials for HIV-1 prevention and treatment.
More recently, a similar approach was applied to ZIKV and led to the discovery of more than 400 antibodies to the ZIKV EDIII (ZEDIII) out of which some were recurring, i.e. found in multiple individuals, and targeting a specific epitope on the ZEDIII (7).
One of these anti-ZIKV antibodies is Z021, a potent ZIKV neutralizer that recognizes an epitope on the lateral ridge of ZEDIII. Besides being a potent ZIKV neutralizer *in vitro*, Z021 is also protective against ZIKV in mice and a combination of Z021 with another monoclonal anti-ZEDIII antibody, Z004, was administered to macaques followed by high-dose intravenous ZIKV challenge which suppressed viremia and prevented the emergence of virus escape mutants (15). Moreover, the passive immunization of pregnant nonhuman primates with these two antibodies could shield their fetuses from the harmful effects of ZIKV (76). These findings indicate that passive administration of human anti-ZIKV monoclonal antibodies could be a safe and efficacious alternative to vaccines for at-risk populations (15). Thus, Z021 was further characterized over the course of this project.
In summary, single-cell antibody cloning is a powerful method that can lead to the identification of antiviral antibodies with translational potential towards clinical applications as well as to key insights in the neutralizing antibody response to pathogens.

1.4 AIMS OF THE THESIS

Flaviviruses are a global threat to humans and the lack of vaccines and treatments for most of them indicates the need to reduce the current impact of flaviviruses on public health. However, this goal requires a better understanding of the characteristics of the human immune response to flaviviruses.
This thesis aims to characterize the cross-reactivity of anti-ZIKV antibodies to TBV as well as to characterize the neutralizing antibody response to POWV in humans. For the latter, using a similar approach as for HIV-1 and ZIKV we aim to

- identify serologic elite responders to POWV from 3 cohorts of individuals that were either exposed to ticks or to MBV,
- discover human antibodies to the E protein of POWV from the memory B cells of elite responders, and
- recombinantly produce and characterize representative antibodies.

In addition, a secondary goal was to expand the library of available flavivirus particles for future studies of neutralization and cross-neutralization.
Altogether, this research bears the potential to discover antibodies that can be developed for diagnostic tests, prevention, postexposure prophylaxis and treatment of patients with POWV disease. Human anti-POWV monoclonal antibodies can furthermore guide the development of POWV vaccines through the identification of neutralizing epitopes.

2 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 CHEMICALS AND REAGENTS

	source	identifier
1 kb Plus DNA Ladder	Invitrogen	Cat#10787018
Ampicillin, 500x	Sigma-Aldrich	Cat#A9393
Bovine Serum Albumin (BSA)	Sigma-Aldrich	Cat#A2153
DENV 1-4 EDIII proteins, no His-Avitag	Dr. J. Keeffe, Caltech	N/A
Deoxynucleotide Triphosphate (dNTP) Solution	Thermo Scientific	Cat#R0192
Dulbecco's Phosphate Buffered Saline (PBS) (1x, 10x)	Gibco	Cat#14190-144, 14200-075
Ethanol, 100%	Decon Laboratories	Cat#2716
Ethidium Bromide Solution, 0.625 mg/mL	Thermo Scientific	Cat#17896
Ethylenediaminetetraacetic acid (EDTA) Solution, 0.5M	Invitrogen	Cat#15575-038
N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES), 1M	Gibco	Cat#15630-080
Kanamycin, 500x	Sigma-Aldrich	Cat#K0254
Lipofectamine 2000	Invitrogen	Cat#11668030
Nuclease-Free Water	Qiagen	Cat#129114
Poly-L-lysine solution, 0.01%	Sigma-Aldrich	P4707
POWV-DTV EDIII protein, His-Avitag	Dr. J. Keeffe, Caltech	N/A
Streptavidin-Allophycocyanin (SA-APC)	eBioscience	Cat#17431782
Streptavidin-Phycoerythrin (SA-PE)	BD Biosciences	Cat#554061
Sulphuric acid	Sigma-Aldrich	Cat#258105
TBEV-EU EDIII protein, His-Avitag	Dr. J. Keeffe, Caltech	N/A
Tris-Acetate-EDTA (TAE) Buffer, 50x	Thermo Scientific	Cat#B49
Trypan Blue Stain (0.4%)	Invitrogen	Cat#T10282
Trypsin-EDTA (0.05%), phenol red	Gibco	Cat#25300054
TWEEN® 20	Sigma-Aldrich	Cat#P7949
Vesphene®IIse Disinfectant Cleaner	STERIS	Cat#6461-08
WNV EDIII protein, no His-Avitag	Dr. J. Keeffe, Caltech	N/A
YFV EDIII protein, no His-Avitag	Dr. J. Keeffe, Caltech	N/A
ZIKV EDIII protein, no His-Avitag	Dr. J. Keeffe, Caltech	N/A

Table 1: Chemicals and reagents.

2.1.2 CELL LINES

	disease	organism	gender	source	identifier
Huh-7.5	hepatocellular carcinoma	Homo sapiens	male	Blight et al., 2002	N/A
K-562	chronic myelogenous leukemia	Homo sapiens	female	American Type Culture Collection (ATCC)	Cat#CCL-243
Lenti-X 293T	N/A	Homo sapiens	female	Clontech	Cat#632180

Table 2: Cell lines.

Huh 7.5 cells were developed in Charles Rice's Laboratory of Virology and Infectious Disease at Rockefeller University and verified by STR (standard tandem repeat).
All cell lines were cultured at 37°C in 5% CO_2, without shaking. A seed-lot system is in place for these highly utilized cell lines.

2.1.3 Composition of cell culture media and BA-1 Diluent

Medium for cell lines.	**source**	**identifier**
Dulbecco's Modified Eagle Medium (DMEM)	Gibco	Cat#11995065
1% Non-Essential Amino Acids	Gibco	Cat#11140076
10% Fetal Bovine Serum (FBS)	GE Healthcare Life Sciences	Cat#SH30396.03
1x Penicillin-Streptomycin	Gibco	Cat#15140122

Table 3: Medium for cell lines.

Freezing Medium (2x)	**source**	**identifier**
40% DMEM	Gibco	Cat#11995065
40% FBS	GE Healthcare Life Sciences	Cat#SH30396.03
20% Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	Cat#D2438

Table 4: Freezing Medium (2x).

BA-1 diluent	**source**	**identifier**
Medium 199	Corning	Cat#10-060-CV
1% BSA	Alfa Aesar	Cat#J65833
1x Penicillin-Streptomycin	Gibco	Cat#15140122

Table 5: BA-1 diluent.

LB medium	**source**	**identifier**
25ug of Luria Broth Base powder	Invitrogen	Cat#12795084
1l of water	N/A	N/A

Table 6: LB medium.

After swirling the Luria Broth Base powder in 1l of water, the liquid is autoclaved at 121°C for 20min.

2.1.4 Commercial Kits

	source	**identifier**
NucleoBond® Xtra Maxi	MACHEREY-NAGEL	Cat#740414.100
NucleoSpin® Gel and PCR Clean-up	MACHEREY-NAGEL	Cat#740609.250
NucleoSpin® Plasmid	MACHEREY-NAGEL	Cat#740588.250
One Shot™ TOP10 Chemically Competent *E. coli*	Thermo Scientific	Cat#404004
Renilla Luciferase Assay System	Promega	Cat# E2810
3,3',5,5'-Tetramethylbenzidine (TMB) Substrate Kit	Thermo Scientific	Cat#34021
Zero Blunt™ TOPO™ PCR Cloning Kit	Invitrogen	Cat#450245

Table 7: Commercial kits.

2.1.5 ENZYMES AND REACTION BUFFERS

	source	identifier
3.1 buffer	New England Biolabs	Cat#B7203S
BamHI	New England Biolabs	Cat#R0136S
Calf Intestinal Phosphatase	New England Biolabs	Cat#M0290S
Cutsmart buffer	New England Biolabs	Cat#B7204S
EcoRI	New England Biolabs	Cat#R0101S
EcoRI buffer	New England Biolabs	Cat#B0101
HotStarTaq DNA Polymerase	Qiagen	Cat#203203
PCR buffer	Qiagen	Cat#203203
PfuUltra buffer	Qiagen	Cat#60038052
PfuUltra Hotstart DNA Polymerase	Agilent Technologies	Cat#600390
SacII	New England Biolabs	Cat#R0157S
SnaBI	New England Biolabs	Cat#R0130S
T4 DNA Ligase	New England Biolabs	Cat#M0202L
T4 DNA Ligase Reaction buffer	New England Biolabs	Cat#B0202S
XhoI	New England Biolabs	Cat#R0146S

Table 8: Enzymes and reaction buffers.

2.1.6 ANTIBODIES

	source	identifier
Anti-Human IgG horseradish peroxidase (HRP) antibody	Jackson ImmunoResearch	Cat#109-035-098
Human recombinant 10-1074	Mouqet et al., 2012	N/A
Human recombinant Z012	Robbiani et al., 2017	N/A
Human recombinant Z021	Robbiani et al., 2017	N/A

Table 9: Antibodies.

2.1.7 OLIGONUCLEOTIDS

All primers were designed by Davide Robbiani according to flaviviral structural proteins (CprME) and synthetized by Integrated DNA Technologies (IDT). Lyophilized primers were reconstituted in nuclease-free water and stored at -20°C.

Primer	region	sequence
p1507	fwd to sequence POWV-LB CprME	AGTGACCGCAAATAAGTCG
p1508	fwd to sequence POWV-LB CprME	AAAAGGTCTGTGGTCATTCC
P1509	fwd to sequence POWV-LB CprME	ACAGGGGATTACTTGGCTG
p1511	fwd upstream of CprME of POWV-LB	ACAGGGGATTACTTGGCTG
p1512	rev to sequence POWV-LB CprME	AGTATTCTCTGGTTTCAGCCG
p1513	fwd to sequence POWV-LB CprME	CGGCTGAAACCAGAGAATACT
p1514	rev downstream of CprME of POWV-LB	ACCGCGGCTCGAGTTAATTAA

Table 10: Primers.

2.1.8 RECOMBINANT DNA

#3491 was synthetized by IDT and contained the following sequence encoding for the POWV-LB CprME region:

CTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAA
TGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTT
GTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGG
CGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCTCTGGCTAACTAGAGAACCcACTGCTT
ACTGGCTTATCGAAATTAATACGACTCACTATAGGGAGACCCAAGCTGGCTAGTTAAGCTATCAACAAG
GAATTCGCGGCCGCCAGGCTATGATGACCACTTCTAAAGGAAAGGGGGGCGGTCCCCCTAGGCGCAA
GCTTAAAGTGACCGCAAATAAGTCGCGACCAGCAACGAGCCCAATGCCAAAGGGCTTCGTGCTGTCGC
GCATGCTGGGGATTCTTTGGCACGCCGTGACAGGCACGGCCAGACCCCCAGTGCTGAAAATGTTCTGG
AAAACGGTACCACTGCGCCAGGCGGAGGCTGTTCTGAAGAAGATAAAGAGAGTTATCGGGAACTTGA
TGCAGAGCCTTCACATGAGAGGGCGTCGCAGGTCAGGTGTGGACTGGACTTGGATTTTTTTGACGATG
GCGTTGATGACCATGGCCATGGCAACCACCATCCACCGGGACAGGGAAGGATACATGGTTATGCGGG
CCAGTGGAAGGGACGCTGCAAGCCAGGTCAGGGTACAAAACGGAACGTGCGTCATCCTGGCAACAGA
CATGGGAGAGTGGTGTGAAGATTCAATCACCTACTCTTGCGTCACGATTGACCAGGAGGAAGAACCCG
TTGACGTGGACTGCTTCTGCCGAGGTGTTGATAGGGTTAAGTTAGAGTATGGACGCTGTGGAAGGCAA
GCTGGATCTAGGGGGAAAAGGTCTGTGGTCATTCCAACACATGCACAAAAAGACATGGTCGGGCGAG
GTCATGCATGGCTTAAAGGTGACAATATTCGAGATCATGTCACCCGAGTCGAGGGCTGGATGTGGAAG
AACAAGCTTCTAACTGCCGCCATTGTGGCCTTGGCTTGGCTCATGGTTGATAGTTGGATGGCCAGAGTG
ACTGTCATCCTCTTGGCGTTGAGTCTAGGGCCAGTGTACGCCACGAGGTGCACGCATCTTGAGAACAG
AGATTTTGTGACAGGAACTCAAGGGACCACCAGAGTGTCCCTAGTTTTGGAACTTGGAGGCTGCGTGA
CCATCACAGCTGAGGGCAAGCCATCCATTGATGTATGGCTCGAAGACATTTTTCAGGAAAGCCCGGCT
GAAACCAGAGAATACTGCCTGCACGCCAAATTGACCAACACAAAAGTGGAGGCTCGCTGTCCAACCAC
TGGACCGGCGACACTTCCGGAGGAGCATCAGGCTAATATGGTGTGCAAGAGAGACCAAAGCGACCGT
GGATGGGGAAACCACTGtGGaTTcTTcGGGAAGGGCAGTATAGTGGCTTGTGCAAAGTTTGAATGCGA
GGAAGCAAAAAAAGCTGTGGGCCACGTCTATGACTCCACAAAGATCACGTATGTTGTCAAGGTTGAGC
CCCACACAGGGGATTACTTGGCTGCAAATGAGACCAATTCAAACAGGAAATCAGCACAGTTTACGGTG
GCATCCGAGAAAGTGATCCTGCGGCTCGGCGACTATGGAGATGTGTCGCTGACGTGTAAAGTGGCAA
GTGGGATTGATGTCGCCCAAACTGTGGTGATGTCACTCGACAGCAGCAAGGACCACCTGCCTTCTGCAT
GGCAAGTGCACCGTGACTGGTTTGAGGACTTGGCGCTGCCCTGGAAACACAAGGACAACCAAGATTG
GAACAGTGTGGAGAAACTTGTGGAATTTGGACCACCACATGCTGTGAAAATGGATGTTTTCAATCTGG
GGGACCAGACGGCTGTGCTGCTCAAATCACTGGCAGGAGTTCCGCTGGCCAGTGTGGAGGGCCAGAA
ATACCACCTGAAAAGCGGCCATGTTACTTGTGATGTGGGACTGGAAAAGCTGAAACTGAAAGGCACAA
CCTACTCCATGTGTGACAAAGCAAAGTTCAAATGGAAGAGAGTTCCTGTGGACAGCGGCCATGACACA
GTAGTCATGGAGGTATCATACACAGGAAGCGACAAGCCATGTCGGATCCCGGTGCGGGCTGTGGCAC
ATGGTGTCCCAGCGGTTAATGTAGCCATGCTCATAACCCCCAATCCAACCATTGAAACAAATGGTGGCG
GATTCATAGAAATGCAGCTGCCACCAGGGGATAACATCATCTATGTGGGAGACCTTAGCCAGCAGTGG
TTTCAGAAAGGCAGTACCATTGGTAGAATGTTTGAAAAAACCCGCAGGGGATTGGAAAGGCTCTCTGT
GGTTGGAGAACATGCATGGGACTTTGGCTCAGTAGGCGGGGTACTGTCTTCTGTGGGGAAGGCAATCC
ACACGGTGCTGGGGGGAGCTTTCAACACCCTTTTTGGtGGtGTTGGATTCATCCCTAAGATGCTGCTGG
GGGTTGCTCTGGTCTGGTTGGGACTAAATGCCAGGAATCCAACGATGTCCATGACGTTTCTTGCTGTGG
GGGCTTTGACACTGATGATGACAATGGGAGTTGGGGCATAATAGTTAATTAACTCGAGCCGCGGTTCG
AAGGTAAGCCT.

	source	identifier
pWNVII-Rep-REN-IB	Pierson et al., 2006	N/A

Table 11: Plasmids.

2.1.9 COLLECTION OF HUMAN SAMPLES

Serum and peripheral blood mononuclear cells (PBMCs) were obtained from consenting donors under protocols approved by the ethical committees of the Rockefeller University (DRO-0898), National Institute of Respiratory Diseases (C16-16),

Columbia University (5847, 6805, 7683), Yale University (IRB HIC 1603017508), Oswaldo Cruz Foundation, also known as FIOCRUZ, (CAAE 63343516.1.0000.5028) and Hospital Geral Roberto Santos (1.998.103). Samples from New York were collected by the Lyme Disease Clinic of Columbia University (from 2009-2019); those from Mexico were obtained in the Spring/Summer of 2016 from residents of Santa Maria Mixtequilla, shortly after a ZIKV outbreak; those from Brazil were collected in Pau da Lima in 2010, following a DENV outbreak. PBMCs of donor MEX 58 were purified by gradient centrifugation with Ficoll and frozen to liquid nitrogen in 90% heat inactivated FBS and 10% DMSO. Serum aliquots were inactivated at 56°C for 1hr and then stored at 4°C before use. All samples were shipped to Rockefeller University for analysis.

2.1.10 CONSUMABLES AND EQUIPMENT

	source	**identifier**
12-Channel VIAFLO II Electronic Pipette, 5-125 µl	Integra Biosciences Corp	Cat#NC0852284
96-well Clear V-Bottom Not Treated Microplate	Corning	Cat#3897
96-well Flat Bottom Assay Plate, White	Costar	Cat#3912
96-well Flat Bottom High Binding Microplate	Corning	Cat#3361, 9018
96-well Half-Area Flat Bottom High Binding Microplate	Corning	Cat#3697
Cell Culture Flask (75cm², 175cm²)	Falcon	Cat#353136, 353118
Centrifuge 5430	Eppendorf	Cat#022620509
Centrifuge tubes (15mL, 50mL, 250mL)	Corning	Cat#430790, 430828, 430776
Centrifuge, J-26XP	Beckman Coulter	N/A
Centrifuge, J6-MI	Beckman Coulter	Cat#360292
Countess™ Automated Cell Counter	Invitrogen	N/A
Countess™ Cell Counting Chamber Slides	Invitrogen	Cat# C10228
Cryogenic Storage Vials	Fisher Scientific	Cat#12-567-501
Deep Well Microplates	Scienceware	Cat#378600001
Easypet® 4421	Eppendorf	N/A
Ecotron Incubator	Infors HT	N/A
FLUOstar® Omega	BMG LabTech	N/A
FLUOstar® Optima	BMG LabTech	N/A
Forma™ Series II Water-Jacketed CO2 Incubators	Thermo Scientific	Cat#3110
Freezer (-20°C)	Fisher Scientific	N/A
Freezer (-80°C)	Thermo Scientific	Cat#ULT20909V3I
Gel Electrophoresis Power Supply, VWR 105	VWR	N/A
Ice Machine	Hoshizaki	N/A
ImageQuant LAS 4000	GE Healthcare	N/A
Isotemp™ General Purpose Water Bath	Fisher Scientific	Cat#FSGPD20
MicroAmp™ 8-Cap Strip	Applied Biosystems	Cat#N8010535
Microplate Washer, AquaMax® 2000	Molecular Devices	N/A
Microwave	Panasonic	Cat# NN-SN797S
Multitron Incubator	Infors HT	N/A
NanoDrop™ 2000c Spectrophotometer	Thermo Scientific	ND-2000C

Nitrogen Container, CryoPlus™	Thermo Scientific	Cat#7404
Owl™ EasyCast™ B2 Mini Gel Electrophoresis Systems	Thermo Scientific	N/A
Pipet-Lite Multi Pipette L12-200XLS+	Rainin	Cat#17013810
Pipette tips (10µl, 20µl, 200µl, 1000µl)	Mettler Toledo	Cat#17002419, 30389267, 17002420, 30389276, 17002410
Pipette tips, 125µl	Integra Biosciences Corp	Cat#NC0043753
Pipettor Solution Basins	Biotix	Cat#730-001
Refrigerator	Fisher Scientific	N/A
Round-Bottom Polypropylene Tubes	Corning	Cat#352059
SimpliAmp™ Thermal Cycler	Applied Biosystems	Cat#A24811
StrataCoolerCryo Preservation Module	Agilent Technologies	N/A
Stripette® Serological Pipets (5ml, 10ml, 25ml, 50ml)	Costar	Cat#7045, 7015, 7016, 7017
Syringe Filter, 0.2µm, 25mm	Pall Life Sciences	Cat#4612
ThermoMixer®	Eppendorf	N/A
UV Transilluminator	Spectroline	Cat#TI-312E
Vortex-Genie 2	VWR Scientific	Cat#58815-234
Weighing machine, NewClassic MS	Mettler-Toledo	N/A

Table 12: Consumables and equipment.

2.1.11 SOFTWARE

	source	**identifier**
MacVector 17	MacVector	http://macvector.com/
Microsoft Office 365	Microsoft	https://products.office.com/
Prism (v8)	GraphPad	http://www.graphpad.com/

Table 13: Software.

2.2 METHODS

2.2.1 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) TO DETECT IGG ANTIBODIES BINDING TO FLAVIVIRAL EDIII PROTEINS

For the screening of serum samples, high binding 96-well plates were incubated with 250ng of flavivirus EDIII proteins in 50µl PBS per well overnight at 4°C. After washing the plates four times with 300µl of PBS with 0.05% Tween 20 (PBS-T) per well, the plates were blocked with 200µl of 1% BSA, 0.1mM EDTA in PBS-T per well for 2hr at room temperature. Upon washes, 50µl of serum samples diluted 1:500 with PBS-T were added. Post 2hr incubation at room temperature, the plates were washed and 50µl of Anti-Human IgG HRP antibody in PBS-T per well were added for 50min at room temperature. After the final washes, the plates were developed using 50µl of TMB substrate per well. The reaction was stopped with 50µl of 1M sulphuric acid per well. The half-maximal binding of the human serum samples and of the recombinant monoclonal antibodies were determined similarly, using serially diluted samples (for serum: 1/3 dilutions in PBS-T, starting with 0,3; for monoclonal antibodies: 1/3 dilutions in PBS-T, starting with 10 µg/ml or 4 µg/ml; 11 dilutions in total).

The half maximal effective concentration (EC50) indicated the antibody concentration needed for 50% of its maximal binding. The 50% binding titer (BT50) represented the reciprocal of the serum dilution that resulted in 50% of its maximal binding. EC50 and BT50 were determined by non-linear regression analysis with Prism software.

2.2.2 Production of luciferase-expressing Reporter Virus Particles (RVPs)

Luciferase-expressing Reporter Virus Particles (RVPs) were produced in Charles Rice's Laboratory of Virology and Infectious Disease at the Rockefeller University, as previously reported (7). In short, $1x10^6$ Lenti-X 293T cells per well were co-transfected with 1μg of pWNVII-Rep-REN-IB and 3μg of the appropriate flavivirus CprME expression construct using Lipofectamine 2000 according to the manufacturer's instructions. After 4-5hr of incubation at 37°C lipid-DNA complexes were removed and replaced with DMEM containing 20mM HEPES and 10% FBS. RVP-containing supernatants were harvested after incubation for 48-72hr at 34°C. The supernatants were filtered through a syringe filter (0.2μm, 25mm) and frozen at -80°C. To determine the dilution to use in the RVP-based assays to achieve $1.5x10^6$ RLU in the absence of serum or antibody, each batch of RVPs was titrated on Huh-7.5 cells.

2.2.3 Neutralization assay using RVPs

Neutralization assay was performed using antibody or sera dilutions and RVPs on Huh7.5 cells (Fig. 6). In detail, 96-half well plates were seeded with 7,500 Huh-7.5 cells per well in 50μl of media and incubated overnight at 37°C. Depending on the RVP stock, the RVPs were diluted 1:2 to 1:16 in BA-1 diluent. 100μl of diluted RVPs were added to 100μl of serially diluted triplicate samples (for serum: 1/3 dilution in PBS-T, starting with final dilutions of 1/600; for monoclonal antibodies: 1/3 dilution in PBS-T, starting with final dilutions of 250 μg/ml or 10 μg/ml; 9 dilutions in total) and incubated for 1hr at 37°C. Next, 50μl of the RVP/antibody mixture was added to each well of cells and incubated for 24hr. The medium was removed and 35μl of lysis buffer per well was added. After freezing at -80°C, the plates were thawed at room temperature. 20μl of lysed cells were used for Renilla luciferase measurement. In accordance with the manufacturer's instructions the Renilla Luciferase Assay System (Promega) was used to read the plates with the FLUOstar Omega luminometer (BMG LabTech).
Neutralization capacity of the serum/antibody was determined by comparison with the luciferase activity of no serum/no antibody controls. The half maximal inhibitory concentration (IC50) indicated the antibody concentration that was needed to inhibit RVP infection by half. The 50% neutralization titer (NT50) represented the reciprocal of the serum dilution that resulted in 50% inhibition of RVP infection. IC50 and NT50 were determined by non-linear regression analysis with Prism software.

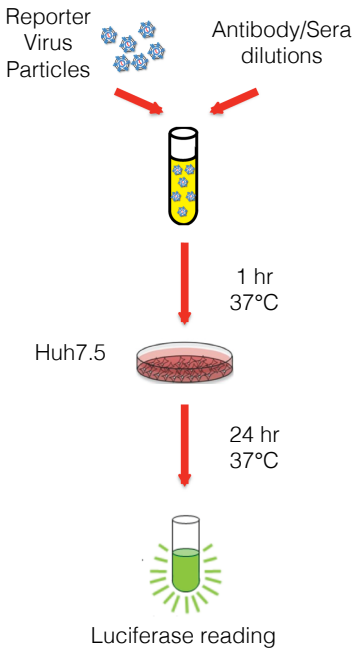

Fig. 6: Neutralization Assay with Reporter Virus Particles (RVPs).
Neutralization assay is performed by incubating antibody or sera dilutions with luciferase-encoding RVPs for 1hr before adding the mix to Huh7.5 cells. After 24hr the luciferase activity is measured by adding the luciferase substrate. It indicates the rate of infection of the cells.

2.2.4 Antibody-dependent enhancement (ADE) Assay using RVPs

96-half well plates were coated with 25µl of 0.01% Poly-L-lysine solution per well for 1hr. After two washes with 60µl of PBS per well, 5000 K562 cells per well were seeded in 25µl of media and incubated overnight at 37°C. Depending on the RVP stock, the RVPs were diluted 1:2 to 1:16 in BA-1 diluent. 50µl of diluted RVPs were added to 50µl of serially diluted triplicate samples (for serum: 1/3 dilution in PBS-T, starting with final dilutions of 1/50; for monoclonal antibodies: 1/4 dilution in PBS-T, starting with final dilutions of 250 µg/ml or 10 µg/ml; 9 dilutions in total) and incubated for 1hr at 37°C. Next, 25µl of the RVP/antibody mixture was added to each well of cells and incubated for 24hr. The medium was removed and 35µl of lysis buffer per well was added. After freezing at -80°C, the plates were thawed at room temperature. 20µl of lysed cells were used for Renilla luciferase measurement. In accordance with the manufacturer's instructions the Renilla Luciferase Assay System (Promega) was used to read the plates with the FLUOstar Omega luminometer (BMG LabTech).

2.2.5 Standard/Overlapping polymerase chain reaction (PCR)

Amplification of DNA by PCR was performed using PfuUltra Hotstart DNA polymerase. 10-20ng of template DNA were diluted in 15µl of nuclease free water and 10µl of the PCR master mix were added per reaction. For overlapping PCR, the two DNA templates were added at equimolar concentrations. The composition of the master mix is shown below (Table 14). Table 15 shows the PCR amplification conditions.

reagent	volume for 1 reaction (in µl)	volume for n reactions in a master mix (in µl)
Nuclease-free water	6	n x 6
10x Pfu buffer	2.5	n x 2.5
10mM dNTP	0.5	n x 0.5
primer A (20uM)	0.25	n x 0.25
primer B (20uM)	0.25	n x 0.25
PfuUltra Hotstart DNA polymerase	0.5	n x 0.5

Table 14: Standard/Overlapping PCR master mix.

step	temperature (in °C)	duration (in min)	cycles
initial denaturation	95	2	1
denaturation	95	0.5	25
annealing	58	0.5	
elongation	72	1 min/kb	
final elongation	72	15	1
	4	infinite	1

Table 15: Standard/Overlapping PCR amplification conditions.

2.2.6 COLONY-PCR

Colony-PCR was used to quickly screen for plasmids containing a desired insert directly from bacterial colonies. Bacterial colonies were picked with a sterile pipet tip and dipped into 10µl of nuclease-free water in a PCR-tube. Each tip was streaked onto a fresh, numbered replicate agar plate, so positive colonies could later be identified. Afterwards, the tip was dipped into 2ml of LB medium with appropriate antibiotics to start miniprep culture. The replicate plate and the 2ml liquid culture were incubated at 30°C overnight.
10µl of the master mix (Table 16) were added to each PCR tube. Amplification of DNA was performed using HotStarTaq DNA polymerase according to specific amplification conditions (Table 17) to lyse the bacteria during the initial heating step of the PCR reaction.

reagent	volume for 1 reaction (in µl)	volume for n reactions in a master mix (in µl)
Nuclease-free water	7.12	n x 7.12
10x Pfu buffer	2	n x 2
10mM dNTP	0.4	n x 0.4
primer A (20uM)	0.2	n x 0.2
primer B (20uM)	0.2	n x 0.2
HotStarTaq DNA polymerase	0.08	n x 0.08

Table 16: Colony-PCR master mix.

step	temperature (in °C)	duration (in min)	cycles
initial denaturation	95	12	1
denaturation	95	0.75	35
annealing	57	0.75	
elongation	72	1 min/kb	
final elongation	72	5	1
	4	infinite	1

Table 17: Colony-PCR amplification conditions.

2.2.7 GEL ELECTROPHORESIS AND IMAGING

Successful PCR amplification or (diagnostic) restriction digestion was confirmed by gel electrophoresis. DNA loading dye was added to the product of the reaction alongside 1kb+ DNA ladder. Both were loaded to a 0.8% agarose gel containing 2 drops of ethidium bromide solution. Gel electrophoresis was performed in 1x TAE-buffer at 100V. The gel imaging was performed using the ImageQuant LAS 400.

2.2.8 GEL EXTRACTION

Gel extraction of DNA fragments was done using the NucleoSpin® Gel and PCR Clean-up Kit.

2.2.9 TOPO® CLONING REACTION

The TOPO® cloning reaction to clone PCR products into pCR™-Blunt II-TOPO® vector was set up using the Zero Blunt™ TOPO™ PCR Cloning Kit.

2.2.10 TRANSFORMATION OF ONE SHOT™ TOP10 CHEMICALLY COMPETENT E. COLI

The protocol of the Zero Blunt™ TOPO™ PCR Cloning Kit for the transformation of One Shot™ TOP10 *E. coli* was applied.
For each transformation 1 vial of One Shot™ TOP10 *E. coli* was thawed on ice and the TOPO® cloning reaction/ligation reaction were added by stirring gently. After heat-shocking the cells for 30sec at 42°C the vials were kept on ice for 2min and 250µl of S.O.C. medium was added to the cells. Next, the cells were shaken at 30°C for at least 1hr. Following a centrifugation for 2min at 3000 xg, 150µl of medium were removed, the cells were resuspended and spread on a pre-warmed LB plate with appropriate antibiotics. Lastly, the plates were incubated overnight at 30°C.

2.2.11 GROWING BACTERIAL CULTURES

To grow a bacterial culture a single colony was picked from a LB plate by a sterile pipette tip and the tip was dropped in 2ml of LB medium with the appropriate selective antibiotic (Ampicillin or Kanamycin). The culture was incubated shaking at 230rpm at 30°C overnight. For minipreparation the 2ml culture was used for plasmid

isolation on the next day. For a maxipreparation, the 2ml culture was, after shaking at 30°C for at least 8hr, added to 250ml of LB medium containing the appropriate selective antibiotic (Ampicillin or Kanamycin). After overnight incubation at 30°C shaking at 230rpm the culture was processed by maxipreparation.

2.2.12 Isolation of plasmid DNA and quality control

Plasmid DNA was isolated using either the NucleoSpin® Plasmid kit for minipreparation or the NucleoBond® Xtra Maxi kit for maxipreparation.
As quality control of the maxipreparation the reconstituted DNA was incubated at 55ºC for 5 to 10min to facilitate the best possible solubilization of the DNA particles. After centrifugation at 3000 xg for 2min to remove potential insoluble DNA the supernatant was transferred into a new tube.
The DNA concentration was measured using the NanoDrop™ 2000c spectrophotometer.

2.2.13 Restriction digestion

To confirm the presence of a correctly-sized plasmid DNA, diagnostic restriction digestions with a total reaction volume of 10µl were performed (Table 18). The reaction was mixed and incubated at the appropriate temperature for at least 30min. The pattern of the fragments after gel electrophoresis indicated whether the plasmid consisted of vector and insert of the expected size.
To isolate an insert from a vector, restriction digestions with a total reaction volume of 30µl were performed (Table 19). After overnight incubation the digestions were heat inactivated at the appropriate temperatures.

reagent	volume for 1 digestion (in µl)	volume for n reactions in a master mix (in µl)
nuclease-free water	6	n x 6
10x reaction buffer	1	n x 1
enzyme A	0.5	n x 0.5
enzyme B	0.5	n x 0.5
DNA aliquot at 200ng/µl	2	

Table 18: Master mix for diagnostic restriction digestions to confirm correct-sized fragments.

reagent	volume for 1 digestion (in µl)	volume for n reactions in a master mix (in µl)
10x reaction buffer	3	n x 3
enzyme A	1.5	n x 1.5
enzyme B	1.5	n x 1.5
DNA aliquot	24	

Table 19: Master mix for restriction digestions to isolate fragments.

2.2.14 Calf Intestinal Phosphatase (CIP) treatment of vectors

CIP treatment was used to remove phosphorylated ends of linearized vectors to prevent their self-ligation. For this, 2µl of CIP was added to a sample after restriction digestion and heat-inactivation of the restriction enzymes. The reaction was incubated at 37°C for 1hr.

2.2.15 Sanger DNA Sequencing

Sanger DNA Sequencing to confirm the presence of the expected DNA and the absence of mutations in the DNA possibly introduced by PCR was performed by Genewiz. For sequence analysis the software MacVector 17 was used.

2.2.16 Ligation reaction

A ligation reaction was set up to ligate a vector and an insert (Table 20). The molar ratio of vector to insert depended on the ends of the DNA (Table 21). After adding and gently mixing all reagents, the ligation reaction was incubated at room temperature for 1hr and, subsequently, at 16°C overnight. A vector-only control was included in parallel replacing the insert by water (Table 20).

reagent	final concentration/amount for a ligation reaction	final concentration/amount for a vector only control
vector	200ng	200ng
insert	Depending on vector to insert ratio (see Table 21)	---
Nuclease-free water	---	Same volume as for insert
T4 DNA ligase buffer	1x	1x
T4 DNA ligase	1x	1x

Table 20: Composition of ligation reactions.

DNA ends	vector: insert ratio
2 staggered ends	1:3
2 blunt ends	1:10
Blunt and staggered end	1:5

Table 21: Vector to insert molar ratio for ligation reactions.

3 RESULTS

3.1 CROSS-REACTIVITY OF ANTI-ZIKV ANTIBODIES TO THE TICK-BORNE FLAVIVIRUSES POWV AND TBEV

3.1.1 Z012 AND Z021 BIND TO THE EDIII OF TICK-BORNE FLAVIVIRUSES

Antibodies to one flavivirus can cross-react with others (77). To determine whether previously characterized anti-ZIKV antibodies can recognize the tick-borne viruses POWV and TBEV, we screened a panel of 42 recombinant human monoclonal antibodies that target the ZEDIII (7). Only two antibodies cross-reacted: Z012 bound to TBEV and Z021 to POWV (data not shown). Dose-dependent binding was confirmed in ELISA (Fig. 7). Z012 and Z021 bound to the ZEDIII with an EC50 of 3.2 and 28.2ng/ml, respectively. Confirming the results of the screening, Z012 bound to TBEV EDIII with an EC50 of 60ng/ml and Z021 to POWV EDIII at 443.4ng/ml (Fig. 7). We conclude that monoclonal antibodies exist that recognize the EDIII of both ZIKV and TBV.

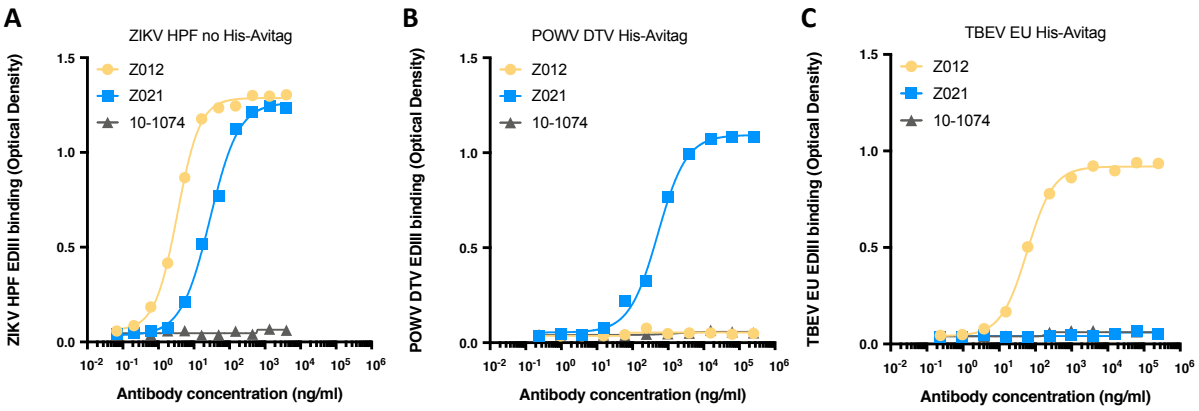

Fig. 7: Binding profiles of Z012 and Z021.
*Concentration-dependent binding of Z012 and Z021 antibodies to the EDIII antigen of ZIKV (**A**), POWV (**B**) and TBEV (**C**), as determined by ELISA. Human anti-HIV antibody 10-1074 was used as a negative control. Graphs represent one out of two independent experiments.*

3.1.2 Z012 AND Z021 ARE POOR OR NON-NEUTRALIZERS OF TICK-BORNE FLAVIVIRUS RVPS

To determine whether these antibodies are neutralizing, we evaluated their ability to prevent infection of luciferase-encoding RVPs that express structural proteins of ZIKV, TBEV and POWV (Fig. 8; see Methods). Z012 and Z021 strongly neutralized ZIKV RVPs with an IC50 of 28.5ng/ml and 0.5ng/ml, respectively. In contrast, Z021 poorly neutralized POWV RVPs (IC50 of 9,733ng/ml) while Z012 failed to neutralize TBEV RVPs even at high concentrations (250,000ng/ml; Fig. 8). Thus, two antibodies that potently neutralize ZIKV are strong binders but poor or non-neutralizers of POWV and TBEV.

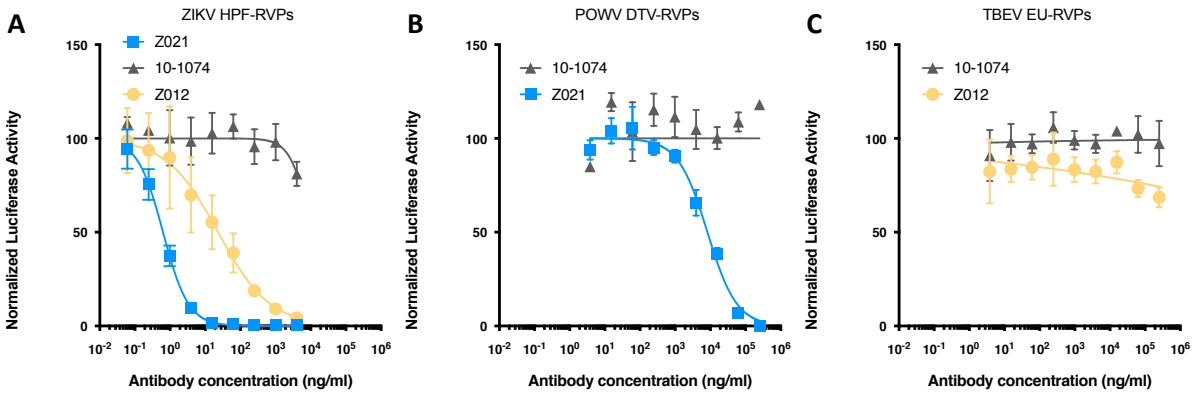

Fig. 8: Neutralization profiles of Z012 and Z021.
*Antibody concentration-dependent neutralization of luciferase expressing ZIKV (**A**), POWV (**B**) and TBEV (**C**) RVPs. Values are normalized to no serum control. Data is presented as mean ± standard deviation (SD) of triplicates. Human anti-HIV antibody 10-1074 was used as a negative control. Graphs represent one out of two independent experiments.*

3.1.3 Z021 ENHANCES INFECTION BY POWV

Antibodies that bind but fail to neutralize can cause ADE of infection (70). To determine whether Z012 and Z021 are enhancing infection of TBEV and POWV, respectively, we evaluated their ADE profile in RVP-based infection assays using K-562 cells (Fig. 9). K-562 is a human myelogenous leukemia cell line that expresses high levels of the activating FcγR and that is unable to efficiently bind flaviviruses and flavivirus RVPs in the absence of antibodies (57). Accordingly, no infection by ZIKV RVP was observed in the absence of antibodies, while addition of Z021 to the cultures induced enhancement of ZIKV RVP infection in a typical bell-shaped ADE curve with an ascending and descending arm (Fig. 9, A): The higher the concentration of Z021 antibody was, the more ZIKV RVP infection was observed (ascending arm) until a maximum of infection was reached and the neutralization effect of the antibody dominated and limited infection (descending arm). This enhancement of ZIKV RVP infection by Z021 was entirely dependent on FcγR binding, because it was abrogated by mutation of the FcγR binding site on Z021 that prevents FcγR binding (Z021-GRLR) (78, 79).
In comparison to ZIKV RVPs, the ADE curve of Z021 for POWV RVPs was shifted to the right and peaked at higher antibody concentrations (Fig. 9, B) which indicates that Z021 enhanced and neutralized POWV RVP infection at higher concentrations than ZIKV RVP infection. This is in agreement with the lower affinity of Z021 for POWV compared to ZIKV. Z012 showed enhancement of ZIKV RVP infection (Fig. 9, C), however, enhancement of TBEV RVP infection above background could not be observed (Fig. 9, D). Nevertheless, the high background signal in the ADE experiments using TBEV RVPs complicates the interpretation of the latter results. Therefore, we conclude that potent anti-ZIKV antibodies such as Z021 that bind POWV EDIII and poorly neutralize POWV RVPs can cause ADE of POWV RVP infection *in vitro*.

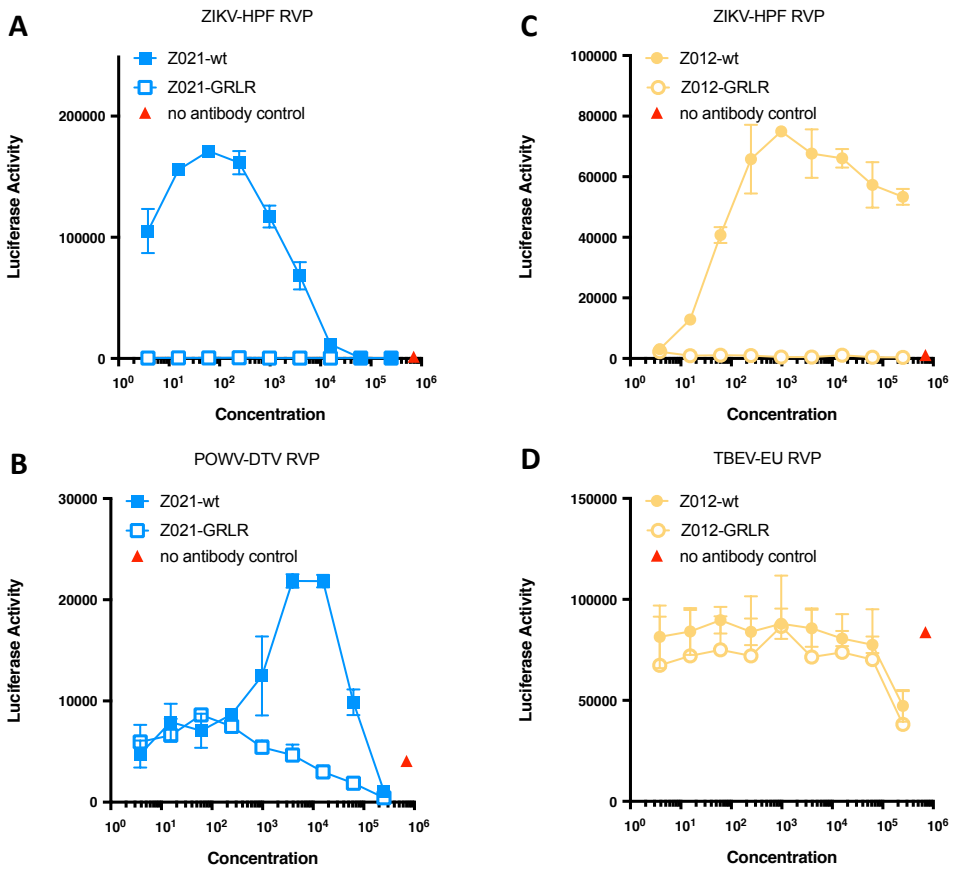

Fig. 9: ADE profiles of Z012 and Z021 using K-562 cells.
*Antibody concentration-dependent enhancement of infection using luciferase expressing RVPs. The profiles of ADE of infection by Z021 (**A** and **B**) and Z012 (**C** and **D**) are shown for the indicated RVPs. Empty symbols are the Fc mutated version of the antibody (GRLR), which is unable to engage FcγR (78, 79). Shown is the absolute luciferase activity. Data are presented as mean ± SD of triplicates. No antibody control is indicated as red triangle. Graphs represent one out of two independent experiments.*

3.2 Serologic evidence for the circulation of POWV in Mexico

3.2.1 Identification of POWV reactive sera from Mexico and Brazil

Symptomatic POWV infection of humans has been reported In the Northeast of the United States (3). To identify individuals who might have antibodies to POWV, we evaluated 591 samples from New York residents exposed to ticks because of confirmed or suspected Lyme disease. As control, we assayed 49 samples from a tropical region in Brazil following a Dengue outbreak in 2010 and 109 samples from Mexico obtained in a dengue endemic, tropical region following a Zika outbreak in 2016. The samples were screened by ELISA for the presence of antibodies binding to the EDIII proteins of POWV (Fig. 10, A). While most samples showed only background reactivity, we identified some strongly reacting sera in all 3 groups. A similar result was obtained in ELISA against TBEV (Fig. 10, B). Thus, a fraction of individuals living in New York or in tropical countries have antibodies to the EDIII of TBV.

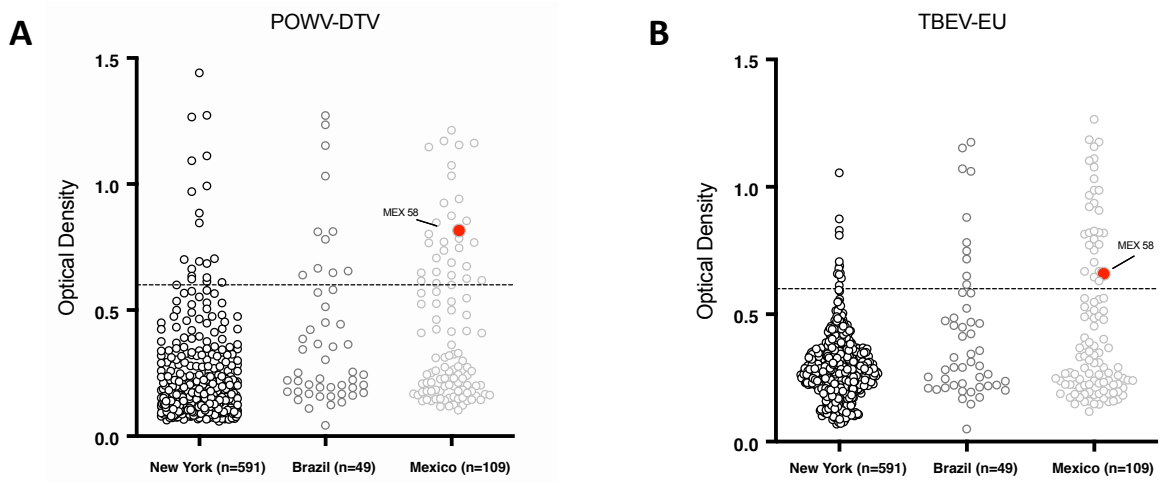

Fig. 10: Screening for serum IgG antibodies binding to POWV and TBEV.
*Sera from New York, Brazil and Mexico were screened in ELISA for IgG binding to the EDIII of POWV (**A**) and TBEV (**B**) at a single serum dilution (1:500). Each dot represents an individual donor. Donor MEX 58 is highlighted in red. Samples with optical density above 0.6 (dotted line) were selected for the neutralization screening.*

To determine whether the sera binding to POWV and TBEV in ELISA also neutralize, we screened them using luciferase-encoding RVPs expressing the structural proteins of these viruses. At the tested serum dilution (1:1200), no neutralization of POWV RVPs was detected in the samples from New York. In contrast, significant neutralization was observed with the tropical samples, particularly those originating from Mexico (Fig. 11, A). Analysis of the same samples for neutralization of TBEV RVPs revealed much weaker neutralizing activity (Fig. 11, B). We conclude that individuals living in Mexico and Brazil have antibodies that neutralize POWV.

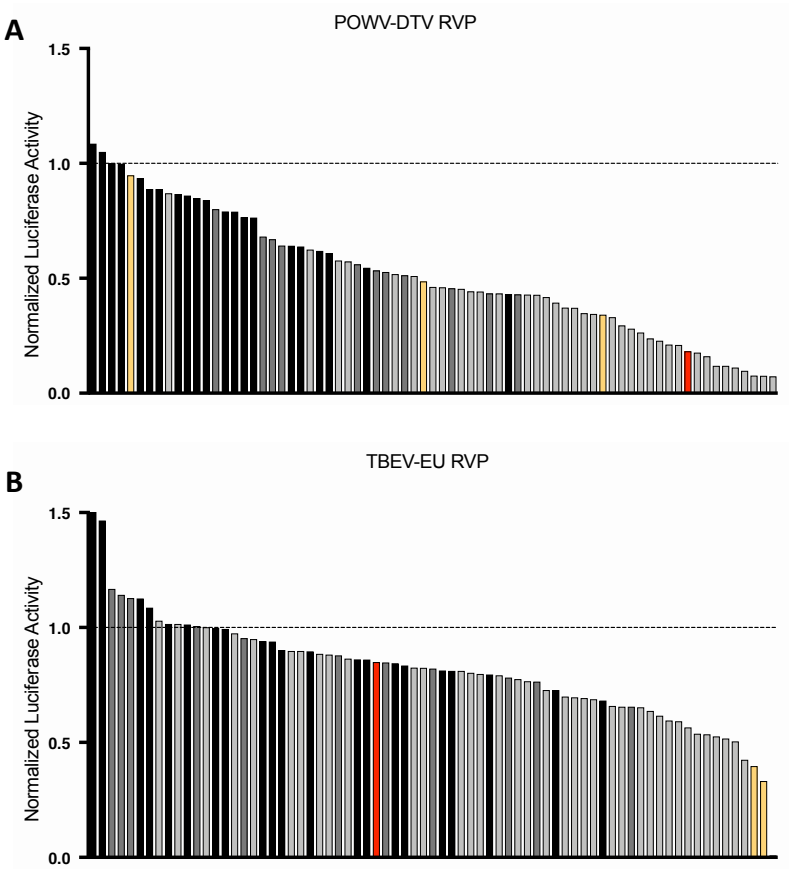

Fig. 11: Screening for antibody neutralization of POWV and TBEV RVPs.
*Selected sera from New York (black), Brazil (dark grey) and Mexico (light grey) were screened at a single serum dilution (1:1200) for neutralization of POWV (**A**) and TBEV (**B**) RVPs. Shown is the luciferase activity relative to no serum control and each bar represents an individual donor. Yellow represents control sera from TBEV vaccinated individuals and red is donor MEX 58.*

To further characterize the screening results, we performed correlation analysis of the Mexican samples (Fig. 12). This analysis revealed that serum antibody binding to POWV and TBEV EDIII were directly correlated (Fig. 12, A) and that a strong binding to POWV EDIII was significantly correlated with strong neutralization of POWV RVPs (Fig. 12, B). However, there was no correlation between the neutralization of POWV and TBEV RVPs (Fig. 12, C). Thus, we conclude that antibodies that neutralize POWV show cross-binding to TBEV but specifically neutralize POWV.

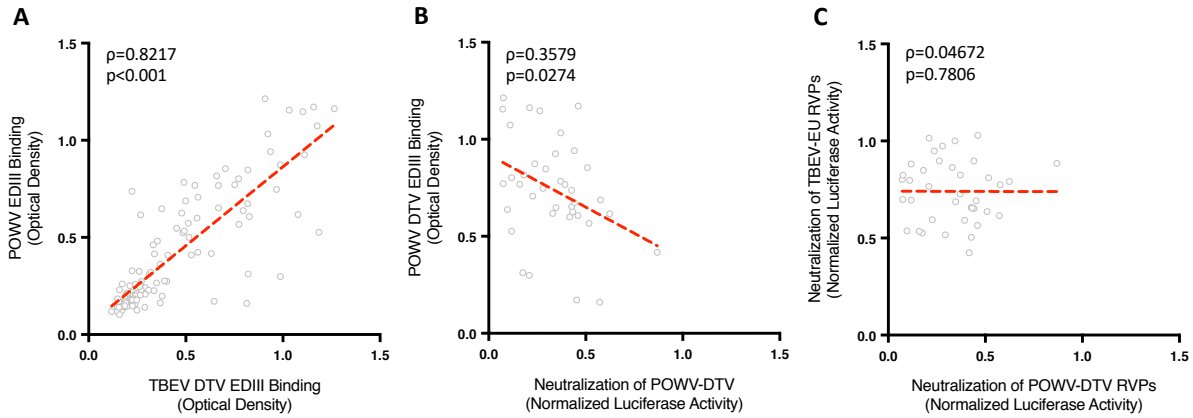

Fig. 12: Correlation analysis of the Mexican samples.
Graphs show the relationships between EDIII binding (measured as optical density) and/or RVP neutralization (measured as normalized luciferase activity). The lower the luciferase activity, the higher the neutralization potency.
***A**: Correlation between serum IgG binding to POWV and TBEV EDIII.*
***B**: Correlation between POWV EDIII binding and POWV RVPs neutralization.*
***C**: Correlation between the neutralization of POWV and TBEV RVPs.*
***A-C:** The p and ρ values were determined with two-tailed paired t test and the two-tailed Spearman r test, respectively.*

3.2.2 CHARACTERIZATION OF SERUM FROM DONOR MEX-58

To confirm the results of the screening, we measured the binding and neutralization properties of individual MEX-58, a donor for which PBMCs were also available (Fig. 13). In ELISA, the sera of MEX-58 and of a control TBEV vaccinated donor bound similarly to the EDIII of POWV and TBEV (Fig. 13, A). In RVP-based neutralization assays, MEX-58 displayed selective neutralization of POWV RVPs with a NT50 of 1,703. In contrast, the TBEV vaccinated control only neutralized TBEV (NT50 of 2,565; Fig. 13, B).
To determine the ADE profiles of MEX-58 we used the RVP system and K-562 cells. Consistent with its neutralizing activity against POWV but not TBEV, serum MEX-58 displayed high enhancement titers to the former compared to the latter. As expected, the inverted pattern was observed with the TBEV vaccinated control (Fig. 13, C). We conclude that although serum MEX-58 recognizes both TBV, it is only effective against POWV and can potentially enhance infection by both TBV.

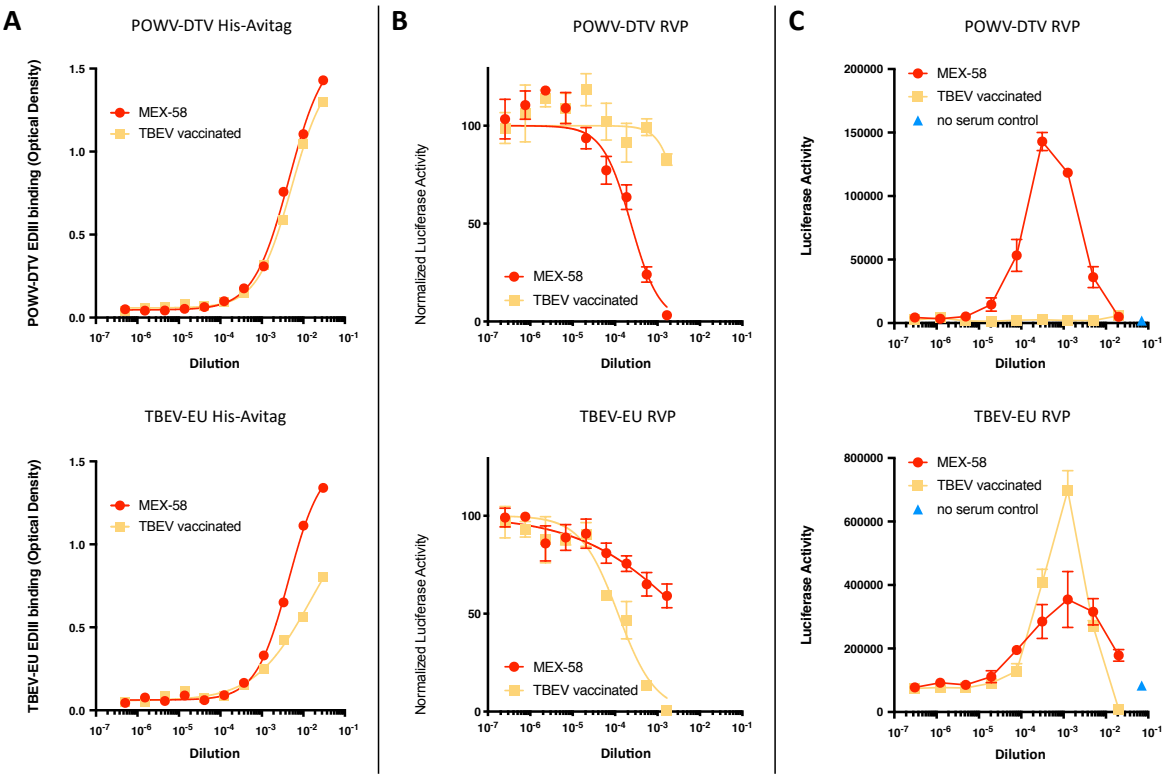

Fig. 13: Characterization of donor MEX-58 and a TBEV vaccinated control.
***A**: IgG binding profile. Serial serum dilutions of MEX-58 and of a TBEV vaccinated control were evaluated for IgG binding to the EDIII of POWV (top) and TBEV (bottom) in ELISA.*
***B**: RVP neutralization profile. Same as in (A) but for RVP neutralization. Shown is the luciferase activity relative to no serum control.*
***C**: ADE profile. Same as in (B) but for ADE of RVP infection.*
***B-C**: Data are presented as mean ± SD of triplicates.*
***A-C**: Graphs are representative of two independent experiments.*

3.2.3 ISOLATION OF POWV EDIII SPECIFIC ANTIBODIES

The discovery of human monoclonal antibodies was performed by Marianna Agudelo, a PhD student in the Nussenzweig laboratory. To discover human antibodies against POWV, virus specific B cells were purified from PBMCs of donor MEX-58 and their antibody genes sequenced using single-cell methods (Fig. 14). IgG switched memory B cells that bound fluorescently labeled POWV EDIII proteins were identified by flow cytometry and sorted as single cells (Fig. 15). Following RNA extraction, reverse transcription, nested PCR and sequencing, anti-POWV antibodies were discovered and subsequently cloned for recombinant expression.
In total, 31 antibodies with productive heavy and light chains were sequenced and 2 families of related antibodies were found for which the genes used in the heavy and light chain genes were similar (Fig. 16). 6 representative antibodies were produced, out of which two antibodies belonging to the same family, P002 and P003, bound to POWV EDIII in ELISA during a screening at a concentration of 10µg/ml (data not shown).

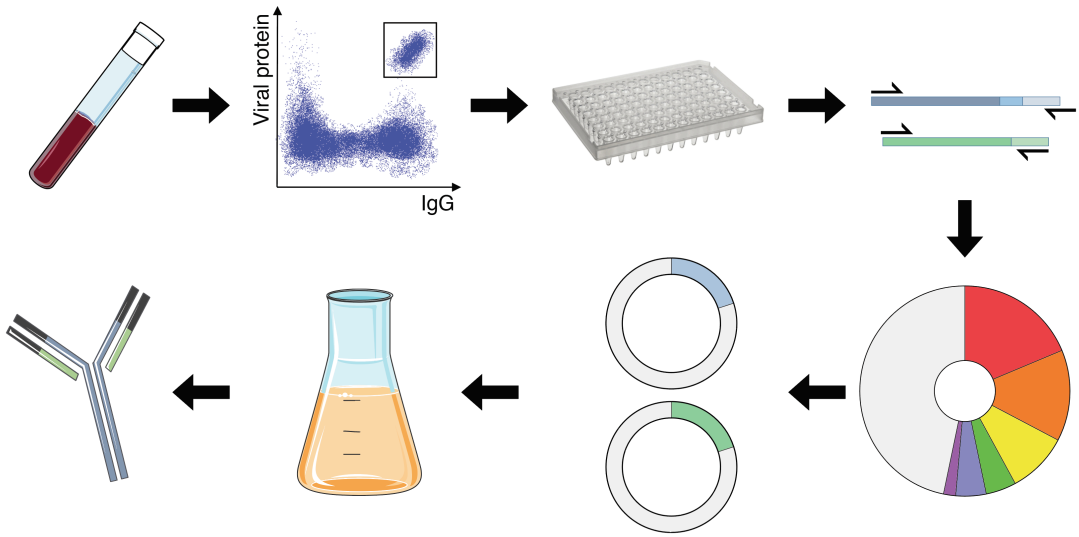

Fig. 14: Single-cell antibody cloning methods to discover human monoclonal antibodies (adapted from Wardemann et al., Science 2003; Scheid et al., Nature 2009).
Single-cell antibody cloning methods established in Michel Nussenzweig's Laboratory of Molecular Immunology at the Rockefeller University for HIV- 1 and ZIKV were adapted to isolate POWV-specific antibodies. Based on the ability to bind to the EDIII of POWV, B cells are isolated and the antibody genes discovered and recombinantly expressed for further analysis.

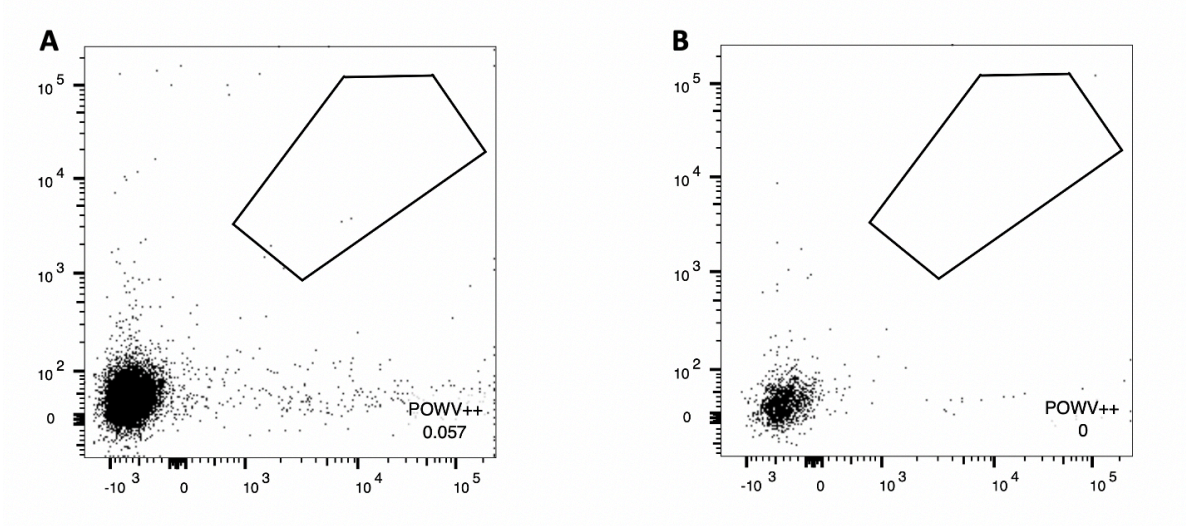

Fig. 15: POWV-specific, IgG+ memory B cells in peripheral blood of Mex-58.
Flow cytometry plots display the percentage of all CD20+ IgG+ memory B cells that bind to fluorescently tagged POWV EDIII baits in Mex-58 (A) and a control (B). On the X and Y axis are the fluorescent signal for POWV EDIII protein bait that was fluorescently labeled with Streptavidin-Allophycocyanin (SA-APC) and Streptavidin-Phycoerythrin (SA-PE), respectively.

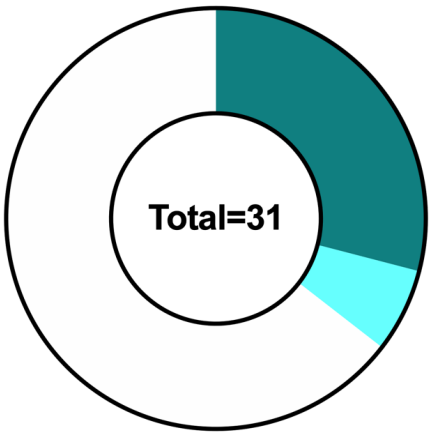

Fig. 16: Anti-POWV Antibody Families.
In total 31 POWV binding human antibodies with productive heavy and light chain sequences were identified from donor MEX-58. The colored areas represent 2 families of related antibodies with similar V genes usage.

3.3 Cross-reactivity of POWV antibodies to other flaviviruses

3.3.1 Binding of POWV antibodies to the EDIII of POWV and other flaviviruses

To characterize the human anti-POWV antibodies P002 and P003, their dose-dependent binding to POWV and other flaviviruses was measured in ELISA (Fig. 17): P002 and P003 bound to the EDIII of both lineages of POWV, ZIKV, WNV and USUV. The particular EC50 values are listed in Table 22. The antibodies failed to bind robustly to the EDIII of all four lineages of DENV, TBEV and YFV (Fig. 17). Therefore, we conclude that P002 and P003 bind to POWV and cross-react to the mosquito-borne viruses ZIKV, WNV and USUV.

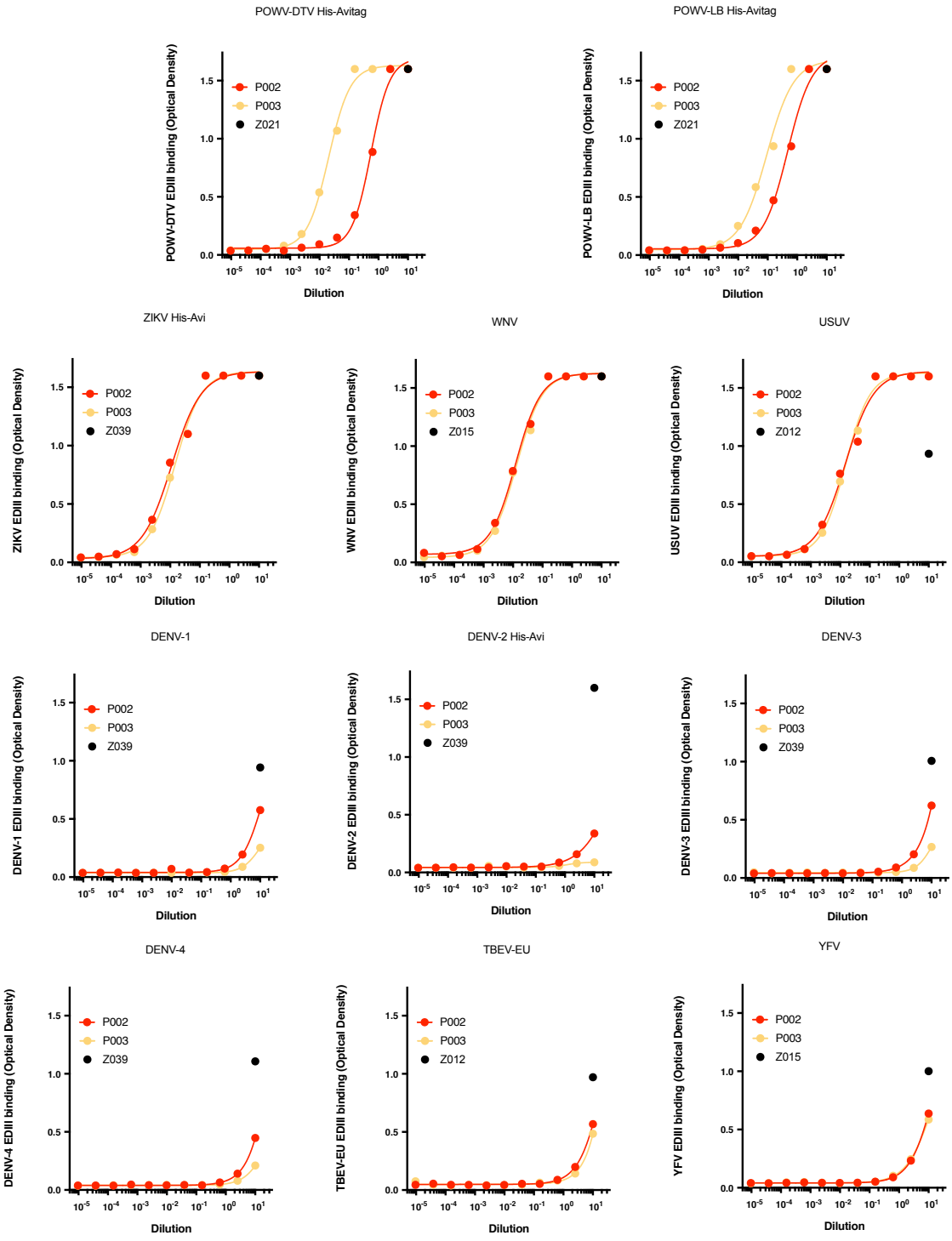

Fig. 17: Binding profiles of P002 and P003 to POWV and a panel of other flaviviruses.
ELISA plots show the concentration-dependent binding of P002 and P003 to the EDIII of POWV and other flaviviruses. The positive controls are indicated as a black dot in each graph. Graphs are representative of two independent experiments.

EC50 (ug/ml)	POWV-DTV	POWV-LB	ZIKV	WNV	USUV
P002	0.3812	0.3522	0.011365	0.013425	0.01277
P003	0.014526	0.056615	0.01656	0.01524	0.01402

Table 22: EC50 values of P002 and P003 to different flaviviruses.
Values are the average of two independent experiments.

3.3.2 GENERATION OF RVP PLASMIDS FOR CROSS-NEUTRALIZATION STUDIES

To improve our ability to study the neutralization, cross-neutralization and enhancement patterns of sera and antibodies, we generated plasmids for the production of RVPs corresponding to 12 different MBVs and TBVs. The plasmids were used to produce RVPs in Charles Rice's Laboratory at the Rockefeller University (see Methods).
In summary, we amplified two overlapping fragments corresponding to the CprME region of each flavivirus. The two amplicons were then joined by combined PCR and cloned into the final expression plasmid (Fig. 18). This 10-step workflow is exemplified in the following subchapter by the step-by-step construction of the POWV-LB RVP plasmid.

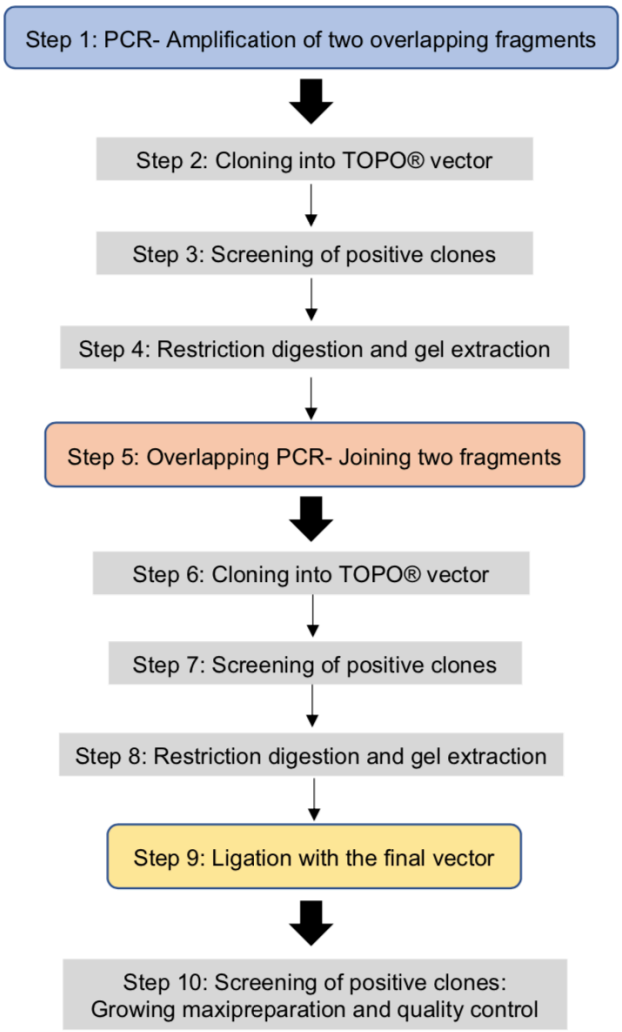

Fig. 18: Workflow for the generation of RVP plasmids.

3.3.3 GENERATION OF POWV-LB RVP PLASMID

Step 1: We have amplified two overlapping fragments of the POWV-LB CprME region by PCR using as template synthetic DNA #3491: For fragment 1 primer p1511 and p1512 and for fragment 2 primer p1513 and p1514 were used. We verified successful amplification of the fragments by gel electrophoresis.
Step 2: After gel extraction of the correct-sized bands from the agarose gel, we cloned the fragments into the pCR™-Blunt II-TOPO® vector and transformed TOP10 cells with this plasmid.
Step 3 + 4: We analyzed positive clones by growing bacterial culture and minipreparation. Afterwards, the isolated plasmid DNA was analyzed by restriction digestion with EcoRI in EcoRI buffer at 37°C. Upon confirmation of the correct-sized vector and insert by gel electrophoresis, we sent the plasmid DNA for Sanger sequencing at Genewiz (primers sp6 and m13F), by which we verified perfect sequences in plasmids #3502-1 and #3503-5.
To release the fragments from the TOPO® vector, we digested #3502-1 with BamHI and XhoI in 3.1 buffer and #3503-5 with EcoRI in EcoRI buffer at 37°C overnight. In order to heat-inactivate the enzymes we incubated the digestions at 65°C for 20min. Upon gel electrophoresis, we gel-extracted the 1.2kb and 1.4kb fragments (#3505 and #3506).
Step 5: To build a DNA sequence encoding for the full POWV-LB CprME, we joined the overlapping fragments #3505 and #3506 by overlapping PCR using primer p1511 and p1514. By gel electrophoresis and extraction, we eluted the PCR product encoding for POWV-LB CprME (#3507).
Step 6 + 7: Next, we cloned #3507 into the pCR™-Blunt II-TOPO® vector and transformed TOP10 cells. Positive clones were screened by minipreparation, diagnostic digestion (EcoRI, EcoRI buffer, 37°C) and sequencing (sp6, m13F, p1507, p1508, p1509). #3507-9 was identified as a perfect clone.
Step 8 + 9: To ligate the insert, consisting of the POWV-LB CprME, with the final vector, we produced both by restriction digestion. We digested #3507-9 (POWV-LB CprME insert in TOPO® vector) and #3473 (final vector including a ZIKV CprME insert) with SnaBI and SacII in Cutsmart buffer at 37°C overnight and heat-inactivated the digestions at 80°C for 20min. We treated the heat-inactivated digestion of #3473 with CIP to prevent the self-ligation of any partially digested, linearized vector. After gel electrophoresis, we gel-extracted the POWV-LB CprME insert (#3509) and the final vector (#3493) and ligated both overnight at 16°C in the presence of DNA ligase.
Step 10: Next, we transformed TOP10 cells with the ligation reaction and screened clones by colony-PCR using primer p1508 and p1512. Positive clones were cultured for maxipreparation and the isolated DNA plasmids were digested twice. In one reaction we digested the plamids with NotI-HF and PacI and in the second with SnaBI and SacII in Cutsmart buffer at 37°C. Clone #3510-42 showed the expected pattern of digestion by gel electrophoresis and was sent for sequencing (p295, p1507, p1508, p1509, p1510, p1528), by which we confirmed a perfect sequence.

3.3.4 GENERATION OF OTHER MOSQUITO- AND TICK-BORNE RVP PLASMIDS

In a similar way as described above for POWV-LB, the plasmids for producing the RVPs corresponding to the three TBEV lineages Sofjin, Western European and Siberian, as well as KFDV, OHFV, LIV, MVEV, LGTV, JEV, USUV and SLEV were constructed and added to our library of flavivirus RVPs (Fig. 19).

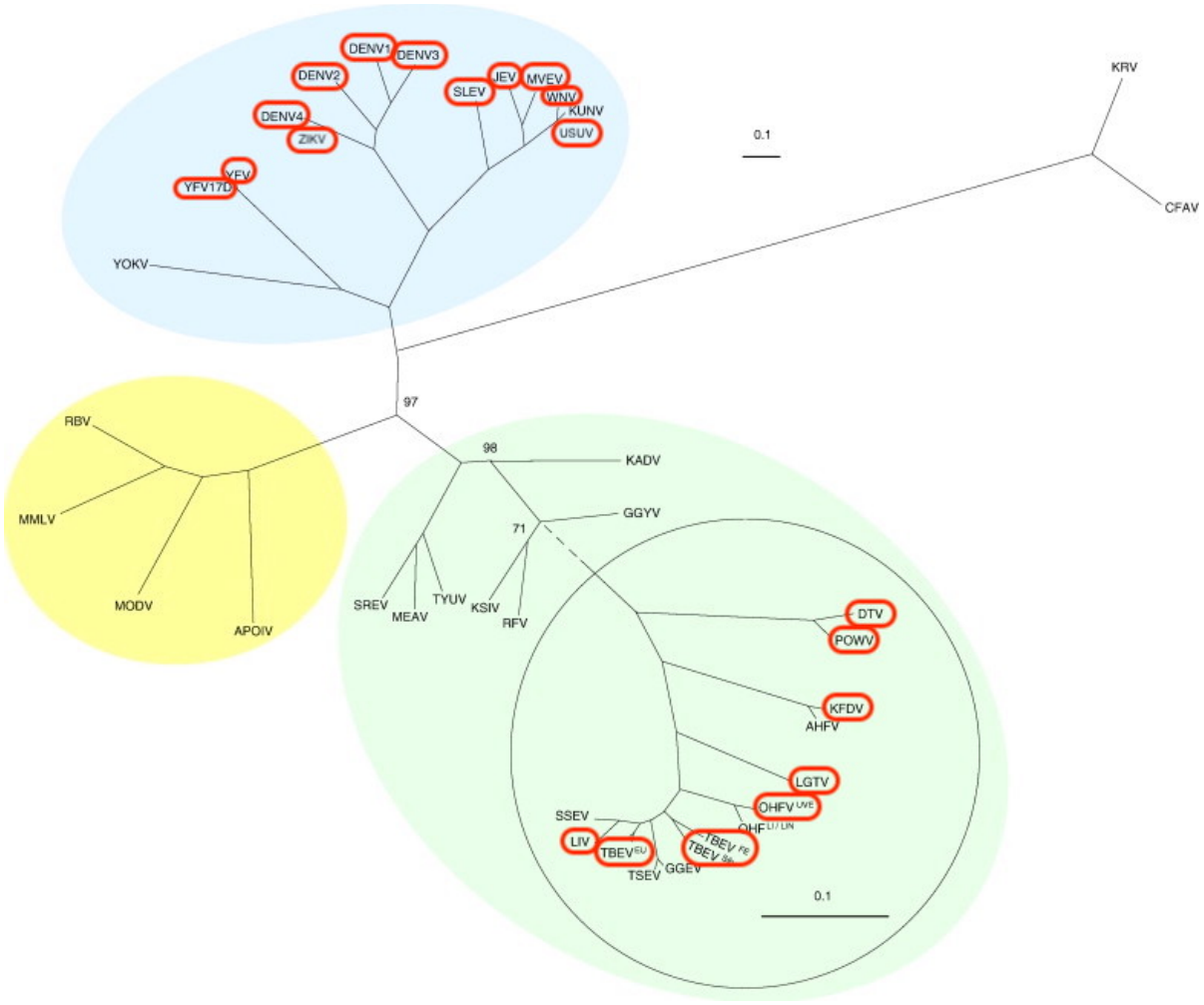

Fig. 19: Library of RVPs available for cross-neutralization studies (adapted from Grard et al., 2007). *The flaviviruses for which RVPs are available for cross-neutralization studies are circled in red. The TBV group is shown in green and the MBV group in blue.*

3.3.5 NEUTRALIZATION OF FLAVIVIRUS RVPS BY POWV ANTIBODIES

P002 and P003 are human anti-POWV antibodies that cross-bind to MBV (Fig. 17, Table 22). To determine their neutralization capacity against POWV and other flaviviruses, we evaluated the ability of P002 and P003 to prevent infection of RVPs corresponding to those flaviviruses for which a strong dose-dependent binding to the EDIII was measured in ELISA. P002 and P003 neutralized POWV-DTV with IC50 of 2.5 and 2.8ug/ml, respectively. In contrast, both antibodies failed to neutralize POWV-LB RVPs even at high concentrations. Also, no neutralization of ZIKV, WNV and USUV RVPs was observed. Unexpectedly, both antibodies appeared to enhance USUV infection *in vitro*,

in a dose-dependent fashion. Thus, we conclude that P002 and P003 do cross-bind MBV but are specific neutralizers for POWV-DTV.

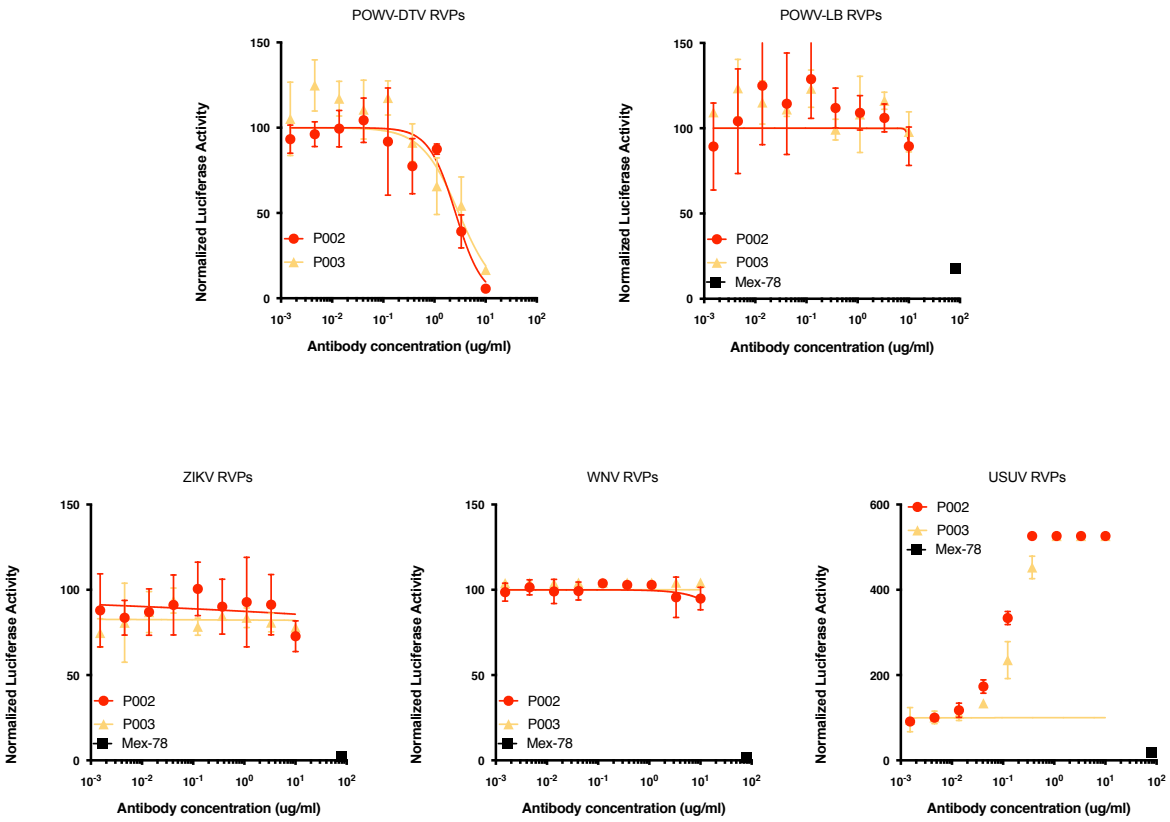

Fig. 20: Neutralization profiles of P002 and P003.
Graphs show the luciferase activity relative to no serum control in the presence of serial antibody dilutions. Data is presented as mean ± SD of triplicates. Sera from donor MEX-78 was used as positive control. Graphs represent one out of two independent experiments.

4 DISCUSSION

Increases in flavivirus disease prevalence and transmission are an important public health concern. Efforts to control these emerging diseases are hampered by the lack of vaccines and treatments for most of them, which in turn first requires a better understanding of the human antibody response to flaviviruses. Therefore, the two goals of the study were to characterize the cross-reactivity of anti-ZIKV antibodies to TBV and to characterize the human neutralizing antibody response to POWV.

4.1 CROSS-REACTIVITY OF ANTI-ZIKV ANTIBODIES TO THE TICK-BORNE FLAVIVIRUSES POWV AND TBEV

Flavivirus infections are known to elicit virus-specific and also cross-reactive immune responses (10, 80). This cross-reactivity and -neutralization of antibodies against multiple flaviviruses is a feature that could potentially be harnessed for vaccines and other therapies but might also promote adverse immune responses. In this context, cross-reactivity of human anti-ZIKV antibodies against TBV haven't been described previously.

Out of a panel of 42 anti-ZIKV antibodies we found two, Z012 and Z021, that strongly bound to TBV, TBEV-EU and POWV-DTV, respectively, but were weakly or non-neutralizing. Cross-reactive antibodies could enhance infection due to increased virus uptake by FcγR mediated internalization of virus-antibody complexes (6, 70).
In agreement with the low neutralizing activity of Z021 against POWV, Z021 showed enhancement for POWV RVP infection at high antibody concentrations. In contrast, no difference from control was seen for Z012 and TBEV RVPs, although the interpretation of this result is complicated by the high level of background infection with control, likely due to the ability of TBEV RVPs to infect K-562 cells through a FcγR-independent pathway.

Implications and future directions

The observation of ADE *in vitro* does not necessarily mean that enhancement of infection or disease will also happen *in vivo*. To evaluate whether Z021 also enhances POWV infection *in vivo* we plan to do experiments in mice. To gain additional insights into the molecular basis of EDIII epitope recognition and cross-reactivity by Z021, we plan to solve crystal structures of complexes of the antigen-binding fragment (Fab) of Z021 with POWV EDIII and compare it to the previously described and published crystal structure of Z021 with ZEDIII (Fig. 21) which showed that Z021 recognizes an epitope on the lateral ridge of the ZEDIII protein (15). A discovery of shared epitopes on the EDIII of ZIKV and POWV could improve our understanding of the mechanisms and relation underlying potent neutralization and cross-reactivity.
This is particularly important because Z021 is a promising candidate as a ZIKV therapeutic: Z021 is protective against ZIKV in mice, in macaques (15) and shielded fetuses of nonhuman primates from the harmful effects of ZIKV, when applied in combination with Z004, another potent anti-ZIKV antibody (76). Therefore, Z021 in combination with Z004 is a candidate ZIKV therapeutic for prevention and the

indication of cross-reactivity and potential enhancement of POWV disease by Z021 is of possible concern.

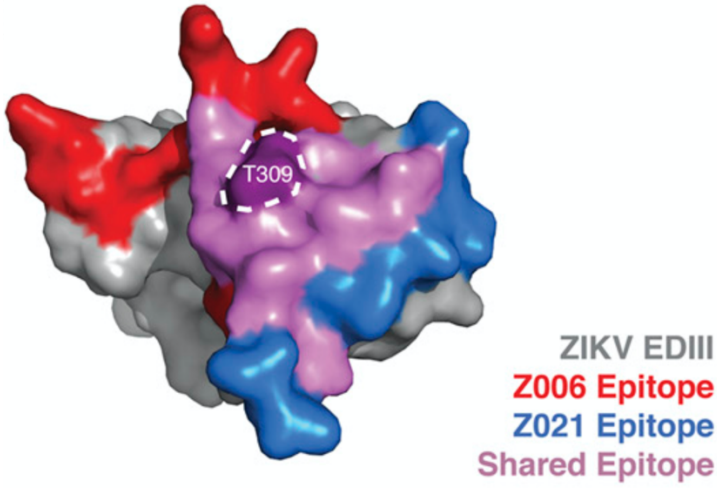

Fig. 21: Epitopes of ZEDIII (from Van Rompay et al., 2020).
"The epitopes of ZIKV EDIII recognized by the Z004-related antibody Z006 (in red), by Z021 (in blue), and by both antibodies (in purple) are shown. Residue T309 is highlighted."(76)

4.2 Serologic evidence for the circulation of POWV in Mexico

POWV is an emerging TBV that can lead to fatal neuro-invasive disease in 10-15% of affected people. Approximately 75% of reported survivors suffer from diverse short- or long-term neurological sequelae (3). However, as of today little is known about POWV and the human antibody response to it. One reason might be that most POWV infections are asymptomatic and thus remain undetected (81). Additionally, neuro-invasive disease can be caused by many different viruses and bacteria that occur in the same or overlapping regions as POWV, some of which may be even transmitted by the same vectors (3). Since no human antibodies to this virus have been described so far, the study of the human antibody response to POWV, and, in particular, the molecular features of human antibodies against POWV, is an important milestone in our understanding of this emerging virus.

Because symptomatic POWV infection of humans has been reported in the Northeast of the United States (3) we have screened 591 serum samples from New York residents exposed to ticks because of confirmed or suspected Lyme disease but we could not identify New York residents from our cohort with strong POWV or TBEV neutralizing antibodies.
Nonetheless, in the ELISA and neutralization screening we stumbled upon the unexpected finding of POWV and TBEV sero-reactivity in tropical Mexico and Brazil where no POWV or TBEV have been reported but instead several other flaviviruses such as ZIKV, DENV and WNV are circulating. The samples originating from Mexico showed not only strong binding but also significant and specific neutralization of POWV RVPs. Analyzing the serum titers from the individual MEX-58 supported the observation of a POWV specific sero-reactivity in Mexico. Further antibody cloning experiments from MEX-58 identified anti-POWV antibodies, two of which (P002 and

P003) neutralize POWV-DTV. These antibodies also recognize other flaviviruses (POWV-LB, ZIKV, WNV and USUV) but they do not neutralize them. Interestingly, both antibodies appear to increase USUV RVP infection *in vitro*.

Implications and future directions

PRNT assays to measure the neutralizing activity of the P002 and P003 antibodies against authentic virus and the resolution of their crystal structures in complex with POWV-DTV EDIII are ongoing. Since these antibodies cross-react with ZIKV and other flaviviruses, it is not possible to firmly conclude which virus elicited them. Similarly, for the neutralizing activity against POWV by the sera, it is not possible to determine whether it was induced by prior exposure to a MBV (or a series of MBV), or by a TBV similar to POWV.

However, the fact that the polyclonal antibodies in serum specifically neutralize POWV-DTV raises the possibility that an unrecognized TBV closely related to POWV may infect humans in the tropics with implications for diagnostics and disease pathogenesis due to cross-reactivity and the potential of disease enhancement by antibodies.

Should this be the case, beside identifying the actual virus, measuring the sero-prevalence to POWV in the population would be valuable. With regard to the putative virus' mode of transmission in tropical regions, that remains to be determined. In Mexico, 26 *Ixodes* species are recognized (82) out of which two are known tick vectors for POWV (*Ixodes cookei* and *Ixodes scapularis*) (30). However, data on the distribution of these *Ixodes* ticks is lacking for Mexico, particularly for the tropical regions. In general, it has been shown that *Ixodes* ticks are particularly sensitive to high temperatures of around 30 °C and water loss (83), with higher temperatures associated with increased mortality, reduced oviposition success and reduced host-seeking activity (84). So, according to species distribution models, *Ixodes* ticks are less likely to be present in tropical areas (84, 85) and other potentially important (tick) vectors would need to be evaluated as candidate vectors for transmission.

Beside potentially identifying a novel TBV in the tropics, future research plans are to study the humoral response of patients with confirmed POWV infection. A few convalescent patients have been contacted and are considering enrolling in our study as soon as the coronavirus disease-2019 (COVID-19) pandemic allows it. From their PBMCs we plan to discover and characterize POWV EDIII specific antibodies, that could one day be used for prevention or treatment of POWV infection.

4.3 CONSIDERATIONS FOR *IN VITRO* RESULTS AS CORRELATE FOR *IN VIVO* NEUTRALIZATION AND PROTECTION

4.3.1 TARGETS OF NEUTRALIZING ANTIBODIES

In order to study the reactivity of sera and antibodies over course of this study we focused on the E protein which is responsible for cell entry of flaviviruses and is, as a consequence, the main target of neutralizing antibodies (62). In this context, antibodies to the EDIII are generally more flavivirus-specific and among the most potent neutralizers (67-69). To evaluate the reactivity and specific neutralizing activity of sera and antibodies, we first measured the binding to EDIII by ELISA, and, subsequently, evaluated the strongly reacting sera in ELISA by *in vitro* neutralization. Finally, to identify monoclonal antibodies we purified virus-specific memory B cells and cloned their antibodies.

Although binding to EDIII appears to be a useful measure to predict the virus neutralizing antibody responses it is important to point out that antibodies to other domains of the virus may contribute to virus neutralization. In fact, both the structures on the viral surface that mediate cell entry and the host cellular factors are not understood completely (86). For example, even though host attachment factors such as mannose receptor, heparin sulfate and phosphatidylserine receptors enhance virion binding to cells, our knowledge about their role in virus entry is incomplete and their function may extend beyond simply facilitating virus attachment (86).
Another important factor to take into account when evaluating the neutralization capacity of antibodies and sera is the general assumption that neutralization occurs after a certain minimum number of antibodies bind to a virion (87). For flaviviruses, estimates suggest that this threshold corresponds to 30 antibodies per virion, but this may vary depending on the antibody (57). Beside the antigen itself, two main factors influence at which threshold the neutralization requirements are met: the antibody affinity as well as the accessibility of epitopes on the virus particle (62). The epitope accessibility is influenced by viral breathing which leads to reversible and non-reversible conformational changes and impacts sensitivity to antibody-mediated neutralization (5). For example, the binding to accessible epitopes on the surface, such as the EDIII protein, may be inhibited. Alternatively, usually inaccessible epitopes for antibody binding may be transiently exposed. Some factors are known to influence viral breathing such as temperature and pH but many aspects of viral breathing are still unknown and assumed to vary across flaviviruses (5). Considering the complex nature of the human organism with its highly variable components and influencing factors, the role of viral breathing *in vivo* complicates the transferability of results that are based on *in vitro* assays focusing on one epitope under standardized settings.

In conclusion, our study evaluated the neutralization potency of antibodies and sera using the EDIII protein which is an important target of neutralizing response to flaviviruses. However, the role of other factors influencing neutralization *in vitro* and *in vivo* were not evaluated. Further studies are needed to provide a more comprehensive understanding of the determinants of neutralization.

4.3.2 GENERATION OF RVP PLASMIDS AND EVALUATION OF RVP BASED ASSAYS

Antibody cross-reactivity due to co-circulation of flaviviruses and vaccines can complicate diagnostics and further be associated with altered pathogenesis leading to disease enhancement (14, 70). To increase our ability to study the (cross-) neutralization and enhancement patterns of sera and antibodies for a wider panel of flaviviruses, we generated plasmids for the production of RVPs corresponding to 12 different MBV and TBV, expanding the library of available flavivirus particles to 21 in total (Fig. 19).

The surface of RVPs resembles the one of viruses, which are composed of viral structural proteins (88). RVPs are nonreplicating and thus they can be used at biosafety level 2 (BSL2), which is advantageous for scientific studies. Furthermore, they encode for luciferase, which simplifies detection of infected cells (89). Additionally, RVP based assays are rapid, low-cost and timesaving compared to conventional PRNT assays that take up to 5 to 7 days for plaque forming and for flaviviruses most often need for work in BSL3 (90). However, RVPs are not the same as infectious virus, and may not fully reflect the situation *in vivo*. Thus, while RVPs are ideal for large-scale screening purposes, key findings need to be verified by *in vitro* and *in vivo* experiments using real virus.

In this context, viral breathing has been shown in several systems to impact neutralization sensitivity (91-94). This is also the case for flaviviruses (5, 95-97) and might be relevant for flavivirus RVPs as well. Additionally, the complex attachment factors and entry receptors that determine the neutralization potency of antibodies for a specific cell type in tissue culture (Huh 7.5 for neutralization assay and K562 in ADE assay) might be cell-type specific and not mirror the complexity of the situation *in vivo* (1). Despite all of this, RVPs are a helpful and safe tool to rapidly and efficiently screen and characterize the neutralizing capacity of antibodies.

4.4 EVALUATION OF APPROACHES ON POWV PREVENTION AND THERAPY

Given the recent emergence of POWV in North America (98) and the likely further increase in cases over the coming years associated with expansion of the tick vector as a consequence of the increasing temperatures (33), the development of effective (preventive) therapies and safe vaccines against POWV will likely become more important. Although many TBV have been known for decades, human vaccines with broad availability are only licensed for TBEV (99). A recent study of TBEV vaccinees demonstrated that, despite possessing a high degree of amino-acid identity with TBEV, POWV is not effectively neutralized by TBEV vaccinee sera (100). Consequently, vaccination with the commercially available TBEV vaccines does not appear to be an appropriate approach for the prevention of POWV infection (100).

Recently, a novel approach in a mouse model was proposed: a messenger RNA (mRNA) vaccine against POWV protected against POWV infection, cross-reacted with other TBV and cross-protected against LGTV (101), a TBV member of the TBEV serocomplex, that is not known to cause human disease (21). However, the durability of the protection

and potential disease enhancement of POWV and related flaviviruses remain a major safety concern for this approach.

In general, there is little control of the quality of antibodies that are induced in an individual by any type of vaccines because the humoral response to vaccination is highly variable and dependent upon many factors such as age, genetics, natural infection, and vaccination history, to name a few. This drawback of non-controllable quality of immune response after vaccination has been observed in TBEV vaccinees (100) and could lead to ADE as it is the case for the DENV vaccine, Dengvaxia® (14).

In contrast to that, monoclonal antibodies can be engineered to circumvent the risk of ADE by Fc mutation by which FcγR cannot be engaged. Additionally, several studies have shown that strongly neutralizing antibodies can block the activity of enhancing antibodies, both *in vitro* and *in vivo* (77, 102, 103), by out-competing the enhancing antibodies for binding on the virion or by binding the remaining available sites on the virion and, thus, dominating the effect on the virion (1).

Potently neutralizing monoclonal antibodies were shown to be protective in several animal models of ZIKV, DENV and TBEV infection when administered before or a short period after infection (6, 59, 60, 76, 104). These results might encourage projects trying to develop therapeutic human antibodies that can be used before and after exposure.
While the current incidence of human POWV infection is low, which makes it difficult to target human populations in need of vaccination and to assess the vaccine's economic feasibility, anti-POWV antibodies might be a practical and cost-effective tool to prevent infection of risk groups for a certain period of time and modify the course of POWV disease.

The discovery and development of neutralizing monoclonal antibodies could not only be useful for passive immunotherapy but also increase our understanding about the molecular mechanism of neutralization and cross-reactivity as well as enhancement. This advance could further inform vaccine development through the discovery of novel neutralizing epitopes. Such an antibody-guided vaccine design has been reviewed recently (105), summarizing previously proposed approaches such as reverse (106) or analytic vaccinology (107), structure-based antigen design (108) or epitope focused vaccine design (109). In Fig. 22 these three main approaches are illustrated: Path 1 shows the production of protective antigens as recombinant proteins, path 2 the engineering of proteins for increased stability if necessary, and path 3 the modification to express or display particular domains or epitopes (105).

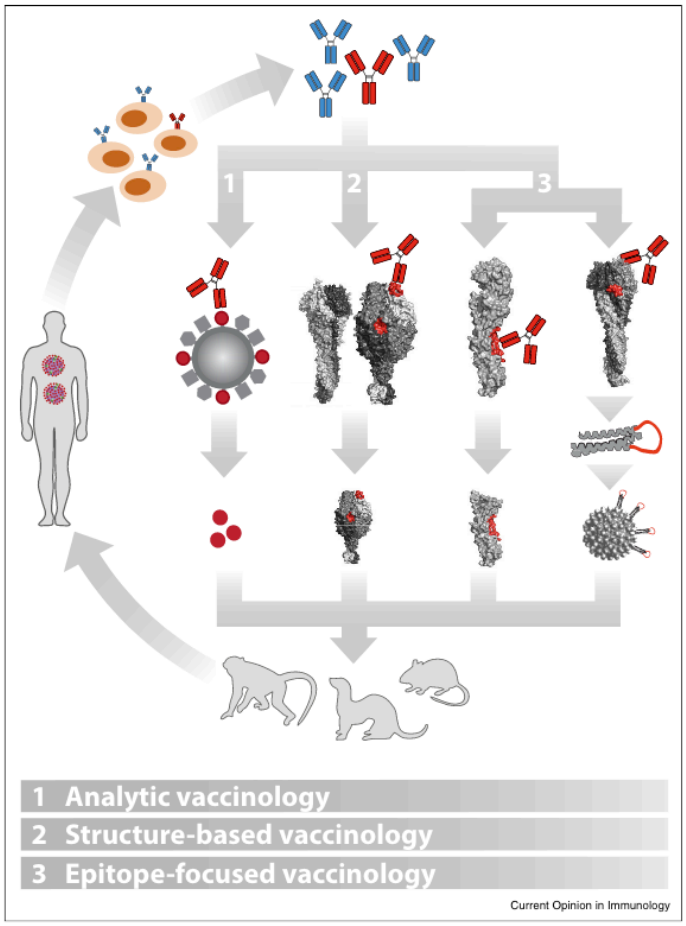

Fig. 22: Antibody-guided vaccine design (from Lanzavecchia et al., 2016).
"Human monoclonal antibodies isolated from immune donors are used to identify protective antigens and epitopes. The antigens discovered from complex pathogens are produced as recombinant proteins (path 1 (…)) and, when necessary, engineered for increased stability (path 2 (…)) or modified to express particular domains or epitopes (path 3 (…))." (105)

Additionally, a panel of POWV specific antibodies could guide the development of highly specific, rapid and inexpensive diagnostic tests for POWV. Identification of POWV infection is currently accomplished by testing the serum or CSF to detect POWV RNA using RT-PCR, or by serological assays to determine the presence of IgM and IgG antibodies using ELISA, IFA and MIA (Fig. 5). In general, serological assays are characterized by potential cross-reactivity which is one of the reasons why POWV infected patients might be diagnosed incorrectly (47). Other assays such as the PRNT are highly specific but labor-intensive, expensive and time-consuming and only show high accuracy a certain period after disease onset, e.g. for ZIKV after day 7 (110). Likewise, RT-PCR is highly specific but expensive and requires multiple labor-intensive sample preparation steps (110). Taking all factors and limitations of POWV diagnostics into account, a highly specific, rapid and cost-effective quantification assay for viral load monitoring at point-of-care settings without the access to specialized equipment and facilities would improve POWV diagnostics immensely and would help to determine the true prevalence of POWV in the population. A similar approach was proposed in 2020 for ZIKV diagnostics by establishing an antibody panel of five antibodies for highly specific detection and differentiation of ZIKV (110).

A concern for antibody-based passive immunization practices using a single monoclonal antibody is the development of neutralization escape mutants. This can be resolved through usage of a cocktail of antibodies that target different epitopes and prevent viral escape from neutralization (1). For example, a recent study showed that the administration of a combination of two monoclonal antibodies, Z004 and Z021, to macaques followed by high-dose intravenous ZIKV challenge reduces viremia and prevents the escape mutants (15).
An innovative concept of antibody engineering to solve the problem of viral escape mutations was recently proposed by constructing a bi-specific antibody comprised of two strongly neutralizing anti-ZIKV antibodies, one targeting EDIII and the other EDII (111). This antibody retained high *in vitro* and *in vivo* potencies and prevented viral escape showing high therapeutic potential (111).

4.5 OUTLOOK

Recent advances in flavivirus immunology have provided new insights into both beneficial and protective as well as potentially harmful antibody responses. However, efforts to put these insights into practice, selectively targeting the protective side of immune responses so that it prevails over the harmful effects, are ongoing but remain challenging. A main challenge is that fundamental mechanisms of the human antibody response to flaviviruses remain incompletely understood, particularly, in terms of (cross-) neutralization of infection, *in vitro* and *in vivo*, protection from disease *in vivo* and ADE.
The lack of knowledge about emerging, lethal pathogens such as POWV, for which therapies and vaccines are lacking, further underlines fundamental gaps in our understanding of many members of the flavivirus genus.
In an interconnected and globalized world that is confronted with an expanding distribution of competent flaviviral vectors and characterized by profound mobility, flaviviruses can spread dramatically across continents. This became apparent by massive ZIKV outbreaks across the Pacific and in the Americas, with up to 4 million infections in 2016 in the Americas alone (6), stressing how a less studied flavivirus that had not caused an outbreak for six decades can become a global health emergency in very short period of time. As stated by the former Director-General of WHO, Dr. Margaret Chan, in the global Strategic Response Plan for ZIKV, “few health threats are local anymore, and few health threats can be managed by the health sector acting alone”(112). In this context, it is up to scientific research to generate data and evidence that is needed to strengthen public health and community guidance and to find safe interventions to prevent, detect and control infections and manage complications (112). In the light of the current COVID-19 pandemic caused by severe acute respiratory syndrome-related coronavirus-2 (SARS-CoV-2), this significant role of science appears to be more relevant than ever before.

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