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Complete absence of linear immunodominant epitope regions recognized by IgG after flavivirus infection and vaccination in whole proteome analyses

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Abstract

English:

The flavivirus genera comprises more than 70 viruses and has an important impact on public health in their endemic and epidemic regions all over the world. Serodiagnosis of specific flavivirus infections is difficult or even impossible, due to the high degree of antibody cross-reactivity. It is however conceivable that serodiagnosis based on one or more individual flavivirus species derived peptides allows to differentiate with high specificity between different viral infections. The primary objective of my doctoral thesis therefore was to map specific immunoglobulin G (IgG) antibody responses after natural flavivirus infections and yellow fever vaccination to identify type-specific signatures of different flaviviruses as well as to study dynamics of cross-reactivity and cross-recognition between them. Therefore, a panel of sera from flavivirus-infected study subjects as well as sera before (D0) and 28 days after (D28) YFV vaccination were tested with the RepliTope™pan-Flavivirus peptide array by the company JPT Peptide Technologies displaying 6253 peptides of various flaviviruses. In general, peptide array technique allows high-resolution, high-throughput, ultra-high-density mapping of linear antibody epitopes. Serum samples prior yellow fever vaccination (timepoint D0) served as negative controls. Data analyses were performed using R-script as well as GraphPad Prism software to further dissect the IgG immune response and to identify potential serodiagnostic peptides. The analysis was focused on detection of potential targets of IgG immune responses located in prM, E, NS1 and NS5 proteins. To summarize the data obtained by heat maps and graphs, our data has shown a high level of individual variation in antibody specificities as well as no flavivirus type-specific IgG peptide recognition signature between different flaviviruses across flavivirus-infected and YFV-vaccinated study subjects. This study points out the difficulties of detecting a flavivirus-specific immune response but gives further insight into a more detailed epitope mapping and understanding of antibody responses in flavivirus infections.

German:

Die Gattung der Flaviviren umfasst über 70 Viren und hat einen wichtigen Einfluss auf das öffentliche Gesundheitswesen in endemischen sowie epidemischen Regionen weltweit. Die Serodiagnostik von speziellen Flavivirus Infektionen stellt sich aufgrund eines hohen Grades an Kreuzreaktivität der Antikörper als schwierig dar. Es ist jedoch vorstellbar, dass Serodiagnostik basierend auf einem oder mehreren individuellen Flavivirus-Peptiden die Differenzierung zwischen verschiedenen Flavivirus Infektionen mit hoher Spezifität möglich macht. Das vorrangige Ziel dieser Doktorarbeit ist die Kartierung spezifischer Immunglobulin G (IgG) Reaktionen nach natürlichen Flavivirus-Infektionen und nach Gelbfieberimpfung, um spezifische Signaturen verschiedener Flaviviren zu identifizieren sowie die Dynamik der Kreuzreaktivität und Kreuzerkennung zwischen diesen zu untersuchen. Daher wurde eine Auswahl an Seren von Flavivirus-infizierten Studienteilnehmer*innen sowie Seren von gesunden Proband*innen vor (Zeitpunkt D0) und 28 Tage nach Gelbfieber Impfung (Zeitpunkt D28) mit dem RepliTope™pan-Flavivirus Peptidarray der Firma JPT Peptide Technologies getestet, welches 6253 Peptide verschiedener Flaviviren abbildet. Im Allgemeinen ermöglicht die Peptid-Array-Technik eine hochauflösende Abbildung von linearen Antikörper-Epitopen. Serumproben vor der Gelbfieberimpfung (Zeitpunkt D0) dienten als Negativkontrollen.

Die Analyse der mit den Peptidarrays gewonnenen Daten umfasste Auswertungs-methoden unter Verwendung von R-Skripten sowie der GraphPad-Prism-Software zur weiteren Aufschlüsselung der IgG-Immunantwort und zur Identifizierung serodiagnostischer Peptide. Die Analyse konzentrierte sich auf den Nachweis potenzieller, spezifischer Erkennungsstrukturen der IgG-Immunantwort, die in prM-, E, NS1- und NS5-Proteinen lokalisiert sind. Unsere Daten zeigten ein hohes Maß an individueller Variation der Antikörperspezifitäten sowie eine fehlende Flavivirus typ-spezifische IgG-Peptid-Signatur bei Flavivirus infizierten und Gelbfieber geimpften Studienteilnehmer*innen bzw. Proband*innen. Diese Studie weist auf die Schwierigkeiten des Nachweises einer Flavivirus-spezifischen Immunantwort hin, gibt jedoch Einblicke in eine detailliertere Epitop Kartierung und ein besseres Verständnis der Antikörper-Antworten bei Flavivirus-Infektionen.

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Abbreviations

(hm) Ab	(Human monoclonal) antibody
Ag	Antigen
ADE	Antibody-dependent enhancement
Arbovirus	Acronym for arthropod-borne virus
BOB assay	Blockade-of-binding assay
CDC	Center for Disease Control and Prevention
CHIKV	Chikungunya virus (Alphavirus)
DENV	Dengue virus (flavivirus)
EDI, EDII, EDIII	Envelope protein domain I, II, III
(Direct) ELISA	(Direktes) Enzyme Linked Immunosorbent Assay
FL	Fusion loop
HI (assay)	Hemagglutination inhibition (assay)
IC-ELISA	Indirect competitive enzyme-linked immunosorbent assay
IEDB	Immune Epitope Database and analysis resource
IFA	Immunofluorescence antibody assay
IFAT	Immune fluorescence antibody test
IgM	Immunoglobulin M
IgG	Immunoglobulin G
JEV	Japanese encephalitis virus (flavivirus)
MAC-ELISA	IgM antibody capture enzyme-linked immunosorbent assay
MFI values	Mean Fluorescence Intensity values
NAAT	Nucleic acid amplification tests
NCBI	National Center for Biotechnology Information database
(RT)-PCR	(Reverse transcription)–polymerase chain reaction
PRNT	Plaque reduction neutralization test
RDT	Rapid diagnostic test
SLEV	St. Louis encephalitis virus (flavivirus)
TBEV	Tick-borne encephalitis virus (flavivirus)

U.S.	United States
VLP	Virus-like particle
WB	Western Blot
WHO	World Health Organization
WNV	West Nile virus (flavivirus)
YFV	Yellow fever virus (flavivirus)
ZIKV	Zika virus (flavivirus)

1 Introduction

Overview of Flaviviruses

The genus *Flavivirus* comprises broadly distributed human pathogens such as Zika virus (ZIKV), Dengue virus (DENV), yellow fever virus (YFV), West Nile virus (WNV), St. Louis encephalitis virus (SLEV), Japanese encephalitis virus (JEV) and tick-borne encephalitis virus (TBEV). They all have an important impact on public health in their endemic and epidemic regions (1).

General public health challenges of flaviviruses are clinically indistinguishable acute fever symptoms, geographical cocirculation especially in the Americas (2,3), under-reporting of asymptomatic infections, (re-)emergence of antigenically similar flaviviruses, lack of specific diagnostic tests and multiplex diagnostics as well as lack of specific antiviral treatments. Additionally, the design of flavivirus vaccinations remains a great task in the future.

Flaviviruses are arthropod-borne viruses and are a subgroup of arboviruses. They are found especially in the (sub) tropics and are mainly transmitted via an arthropod vector such as mosquito (*Aedes and Culex spp*.) or in TBEV via ticks (*Ixodes spp.)*. The mosquitoes *Aedes aegypti* and *Aedes albopictus* serve as vectors of various globally important arboviruses, especially YFV, DENV, and Chikungunya virus (CHIKV), and they mainly target mammals. *Culex* mosquitoes are ornithophilic with birds as their primary vertebrate host and mainly transmitting WNV, JEV, SLEV. Even in Europe, flavivirus infections emerged as Dengue virus cases in Spain, France, and Italy as well as few recurring West Nile virus cases scattered across Europe in recent years have shown.

The incubation period of most arboviral infections ranges from several days to up to two weeks and infections are usually asymptomatic or self-limiting. When symptoms occur, they often consist of flu-like symptoms. Still the correct diagnosis can be crucial because of the potential risk for complications such as viscerotropic and hemorrhagic disease in DENV, ZIKV and YFV as well as neurotropic disease in TBEV, WNV and JEV (4,5). Besides reliable and specific flavivirus diagnostic tests are essential for disease surveillance and disease control.

Currently, there is no cure or a specific anti-viral drug against flavivirus infection available, however effective vaccines for YFV, TBEV and JEV were implemented worldwide. Additionally, a tetravalent live-attenuated vaccine (Dengvaxia®) is accessible under preconditions in some countries including the U.S. (6).

The flavivirus genome is an enveloped, single-stranded positive-sense RNA of ca. 9000 to 12000 nucleotides that encodes ten proteins. Three of them are structural proteins (C, E, prM/M) and seven are non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5).

Serological cross-reactivity and primary vs. secondary flavivirus infection

The greatest problems of diagnostics and surveillance in (commercially) available serological tests (i.e. PRNTs, ELISAs, or other immunoassays) nowadays are that they still lack sensitivity and/or specificity during or after infection (5,7–16) but also after vaccination (17–23).

False-positive results occur due to high serological cross-reactivity among the different flaviviruses, previous immunogenic exposure due to travel or infections (known as secondary flavivirus infection) especially in flavivirus cocirculating areas and flavivirus vaccinations such as the YFV, TBEV or JEV vaccine (24). The differentiation of primary and secondary flavivirus infection based on the detection of antibodies (Abs) is still a challenge (25–27). Besides the protective effect of Abs elicited after natural infection and vaccination, they are also involved in infection enhancement mainly in secondary DENV and ZIKV infections, known as the not yet fully understood antibody-dependent enhancement (ADE) (28,29). Furthermore, WNV Abs in human sera increased ZIKV infection in vitro (30,31) while on the contrary, Abs developed after ZIKV infection enhanced inter alia WNV and SLEV infection (32). ADE effects of the attenuated YFV 17D strain were demonstrated in humans prior immunized with the inactivated JEV vaccine (33). Additionally, higher specificity was observed in tests with recombinant antigens (mostly E, prM, NS1) in ELISA compared to use of whole virus (11–13,26,34–38).

Epitope mapping

Therefore, one current approach in research of arboviruses is to find specific small protein regions targeted by type-specific Abs to find a flavivirus-type-specific immunogenic signature, also known as B cell epitope mapping. Most reports in literature examine the identification of linear epitopes, which are basically short, continuous amino acid sequences. This focus on linear epitopes can be partially explained by the fact that many neutralizing antibody epitopes in proteins are quaternary or conformational according to various epitope prediction databases/tools, meaning that they are difficult to pin down on any type of diagnostic tool or array. Generally, protein epitopes can be grouped into discontinuous/conformational epitopes, with a sound native protein structure and continuous/linear epitopes with consecutive overlapping synthetic peptides comprising the whole primary structure of the antigen (39).

Functional epitope mapping can be achieved using four different techniques: competition methods (in immunoassays), antigen fragmentation methods (in liquid chromatography; mass spectrometry), modification methods (in phage display), and methods using synthetic peptides or peptide libraries (in peptide arrays). Mostly, their use is to detect Ab binding to antigens/epitopes in a whole protein, antigen fragments, synthetic peptides or recombinant antigens (40).

These type-specific linear and/or conformational B cell epitopes targeted by the immune system can be analyzed by two different approaches: either by laboratory techniques, for example by functional or structural methods, or by theoretical, computational tools predicting potential epitopes used in silico analysis.

1.1 Viral structure of flaviviruses

The family of *Flaviviridae* can generally be divided in four genera naming flavivirus, Pestivirus (only epizootic diseases), Pegivirus and Hepacivirus (Hepatitis C virus). The flavivirus genus can further be divided into 23 viral subtypes and further separated into 12 groups based on phylogenetic analysis of the NS5 gene (41). It is composed of viral serocomplexes based on cross-neutralization by polyclonal Abs, which is generally not observed between different serocomplexes (42).

There is the Zika virus (ZIKV); the yellow fever virus (YFV); and tick-borne encephalitis virus (TBEV) with its own serocomplexes, while four serotypes of Dengue virus (DENV) are assigned to the DENV serocomplex (DENVI-IV). The West Nile virus (WNV) and Japanese encephalitis virus (JEV) are part of the JEV serocomplex.

In general, the whole family of flaviviruses share the same particle structure and basic organization in their genome. The flavivirus genome encodes a polyprotein which is cleaved by host and viral enzymes, yielding in 10 proteins [Figure 1].

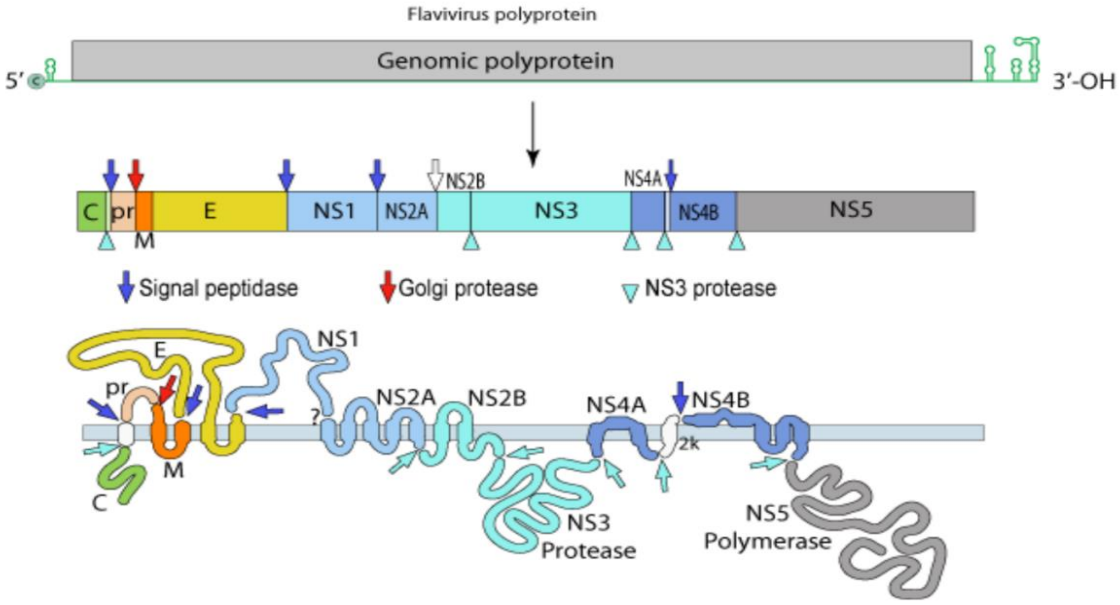


Figure 1 The flavivirus genome organization. The whole flavivirus (polyprotein) genome is translated and further processed via co- and posttranslation by proteases into mature viral proteins. Figure taken from (43).

The genus flavivirus is antigenically and structurally quite similar which is an important factor in lack of good specificity in serological tests: At polyprotein level, the highest sequence identity (55.1%-56.3%) was found between ZIKV and DENV (studied flaviviruses: DENV I-IV, ZIKV, WNV, YFV, TBEV, JEV) (44).

The E proteins in ZIKV and DENV are genomically close, so it was thought to include ZIKV as a 5th serotype in the DENV family (11), although the rest of the genome Zika virus is closer to some of the other mosquito-borne flaviviruses (44).

All in all, most of the information concerning the epitopes which are targeted by human Abs have been analyzed in studies with mosquito borne DENV, WNV, YFV, and JEV.

The numerical superiority of literature available on the IEDB epitope database in 2016 for flaviviruses described DENV epitopes (90%). At the level of antigens, the envelope (E) protein is dominating, presenting over 80% of all Ab targets (44).

All 10 proteins of the flavivirus (polyprotein) genome play different roles in the flavivirus life cycle [Figure 2].

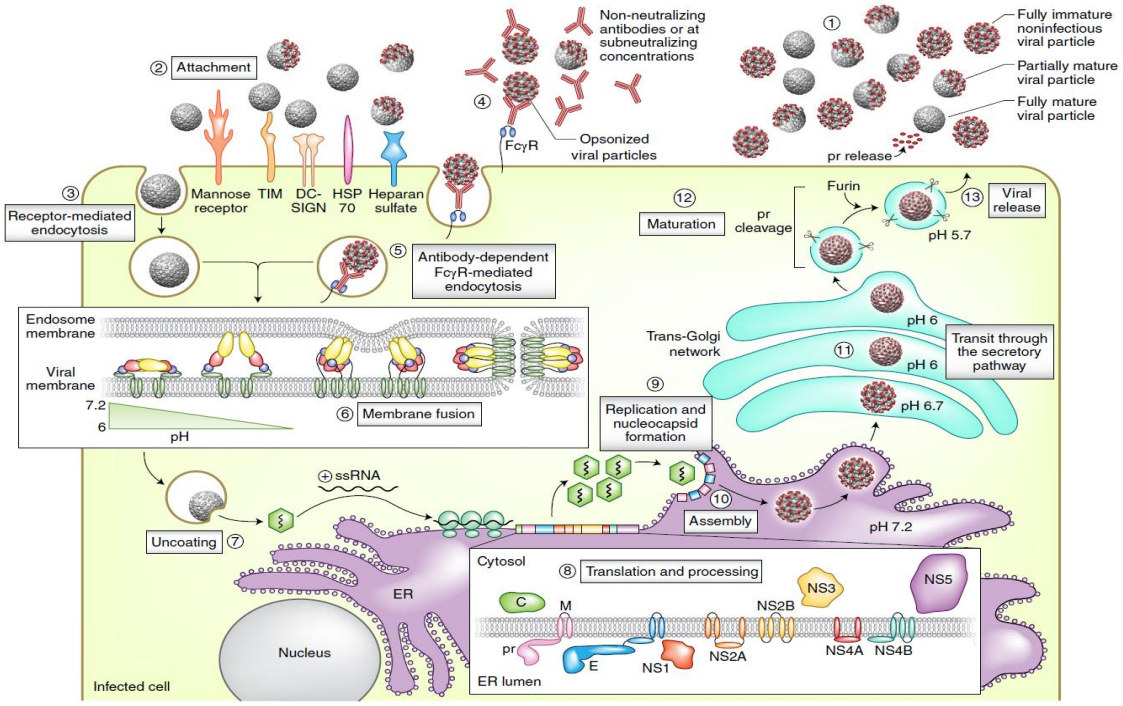


Figure 2 Overview of the life cycle of flaviviruses. 1. Flavivirus particles are present in a fully mature, partially mature or fully immature state. 2. Via interaction with attachment factors, the virus binds to surfaces of susceptible cells 3. Endocytosis and internalization through interaction with secondary receptors 4+5. Alternatively, when non-neutralizing antibodies are present, endocytosis and internalization of opsonized viral particles. 6. Acidification of endosome inducing E dimerization and fusion of virus and endosome membrane 7. Uncoating and release of nucleocapsid in cytosol 8+9.Translation and processing with subsequent replication and nucleocapsid formation 10. Endocytosis into the endoplasmic reticulum (ER) lumen 11. Transit of immature viruses via secretory pathway in Golgi network 12. Maturation mediated by furin protease-mediated cleavage of prM triggered by decreasing pH of Golgi complex. 13. Release of mature pr out of the cell.
TIM: T cell immunoglobulin mucin; DC-SIGN: dendritic-cell-specific intracellular adhesion molecule-3-grabbing non-integrin; HSP: heat-shock protein; ssRNA: single-stranded RNA; C: capsid protein; M: membrane protein.
Figure taken from (45).

The E protein can be further divided into different domains (EDI, EDII, EDIII) (45). It is thought to be highly involved in target cell interaction, inducing immune responses and production of neutralizing Abs (46). While the EDI region mainly serves as a stabilizer of the protein, EDII consist of a fusion loop whose amino acid sequence is highly conserved across flaviviruses.

Therefore Abs recognizing the fusion loop and the neighboring EDII domain are broadly cross-reactive and critical for virus entry (47–50). Nevertheless, the domain has shown to play a major role in DENV infection Ab response (47,48,51), but only a minor role in other flavivirus infections (18,20).

Abs to EDIII have been shown to have the greatest potential for differentiation of a serotype-specific immune response, which was demonstrated in studies with virus-specific epitopes located in recombinant DIII peptides (34–36,52), as well as in a ZIKV EDIII-based ELISAs (53,54). Additionally, the great majority of mAbs (90%) that bound EDIII isolated from ZIKV- or DENV-infected patients were highly specific for ZIKV or DENV E protein (49). However, serum depletion experiments with human DENV-immune serum reveal that EDIII-specific Abs only play a subordinate role in overall antibody reactions and neutralization as well (36,47), also seen in analyses of WNV-infected patients (55,56) and post YFV vaccination (18).

Specific anti-NS1 Abs are involved in protective immunity and important for diagnostic due to its high immunogenicity caused by secretion during viral replication (57). The NS1 protein has been shown to be an important infection marker because soluble NS1 (sNS1) is released into the bloodstream during the acute phase of illness from infected cells, sometimes even before onset of symptoms, and accumulates at high levels of up to 50 µg/ml in DENV (58–60). The development of NS1-based diagnostical assays like ELISA as an alternative to mainly E protein based assays has accelerated in recent years (12,37,61–63).

1.2 Diagnostics of flaviviruses

In general, flavivirus diagnostics tools can be divided into two groups: 1) tools to detect the virus itself via virus isolation of live virus, viral nucleic acid testing, viral antigen detection and 2) tools to detect the immune response after exposure to the pathogen (via detection of virus-specific Abs like IgM or IgG). The selection of the diagnostical assay is mainly dependent on the testing objective and on the timing of sample collection (2) [Figure 3].

Serodiagnosis in clinical practice is based on the detection of IgM and IgG Abs mainly via ELISA-, Immunoblot (Western Blot), Immunofluorescence and virus neutralization test, but also on the detection of virus genome in RT-PCRs especially in early stages of infection, when IgM or IgG Ab responses are not yet measurable (2,64–66).

Besides the role of Abs in disease and pathogenicity, they can also serve as biomarkers for past viral exposure (28). RT-PCR and direct detection of virus in the bloodstream are dependent on the short acute viremic phase of arbovirus infections and is therefore often not achieved in clinical routine. Nowadays one step RT-PCR assays targeting the E gene, NS1, NS3, NS4B, and NS5 genes mostly for ZIKV diagnosis are available (14).

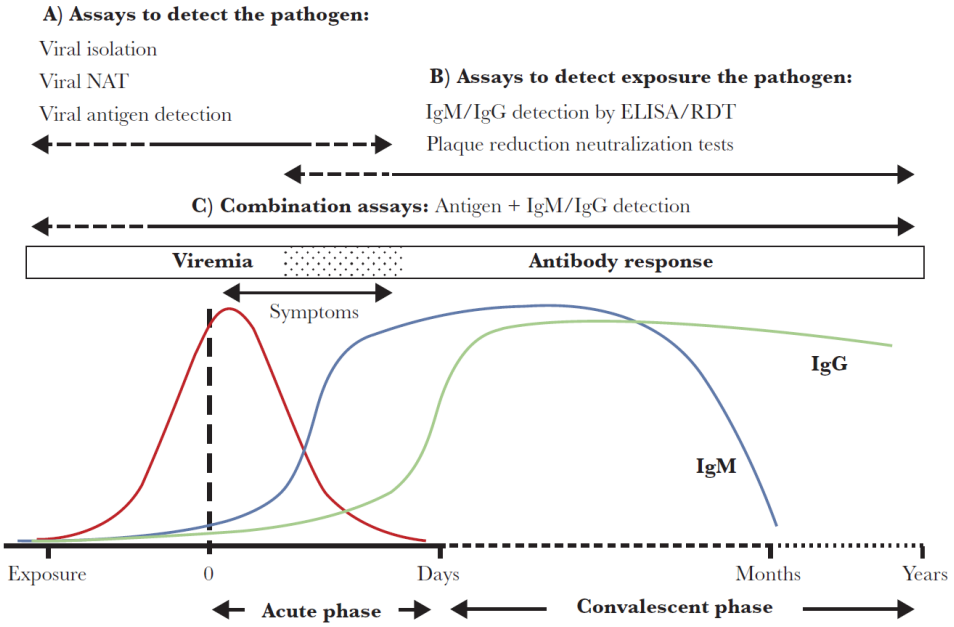


Figure 3 Schematic representation of the typical kinetics of flavivirus infections. In arboviral infections, viremia (red line) usually occurs before onset of clinical symptoms (0) and continues for a few days after onset of symptoms. A: During the acute phase, flavivirus infections are most successfully diagnosed by viral isolation, nucleic acid amplification tests (NATs, e.g. RT-PCR), and antigen detection assays (i.e. the Dengue virus NS1 assay) B: During course of infection, the immune response against the pathogen consist of the production of viral-specific IgM mostly within first week (IgM; blue line) and IgG (IgG; green line) antibodies. While IgM Abs last for several months, IgG Abs can persist for a few years. Another possibility is to perform a quantitative PRNT assay measuring virus-specific neutralizing Ab titers or a hemagglutination inhibition (HI) assay (obsolete).
C: Combination assays that detect either antigen (DENV NS1) and IgM/IgG Abs. ELISA: enzyme-linked immunosorbent assay); RDT: rapid diagnostic test. Figure taken from (2).

The general problem with detection of viremia is that the highest levels exist before beginning of symptoms and decline rapidly in the early acute phase. However, the detection of DENV nonstructural protein-1 (NS1) antigen is achieved with comparable frequency and duration to Dengue viral RNA in serum.

In comparison to the ELISA and IFA (immunofluorescence assay) technique, which detect a wide range of antiviral mAbs, plaque reduction neutralization tests (PRNTs) are quantitative assays measuring virus-specific neutralizing Ab titers for various flaviviruses, to which the tested person could have been in contact with (66).

In a large recent review, various ZIKV ELISAs showed a generally higher specificity than DENV ELISAs. This can be potentially explained by the analysis, that ZIKV ELISAs commonly use NS1 as viral antigen, while DENV ELISAs commonly use full viral particles or the E protein. However, diagnostical results can be tampered by cross-reacting Abs in all individuals who have been infected with or vaccinated against flaviviruses prior testing (7,10,28,67,68). Nevertheless, the PRNT is called the gold standard in convalescent sera sample diagnostic (66). However, it has a turnaround time (TAT) of days to weeks (average 3–7 days per sample), is expensive, labor-intensive and requires trained technicians. The immunofluorescence assay uses fluorophore-conjugated antispecies IgM or IgG to detect virus-specific Abs with high level of cross-reactivity (41).

Serological methods are preferred in the convalescent phase of infection, although paired acute and convalescent phase sera are needed to enable differentiation between present and past infections. A huge number of studies have been conducted in recent years, which focused on Ab detection (IgM and/or IgG) ELISAs to identify a specific immunogenic signature to distinguish between different arboviruses. In 2021, Fumagali et al. reviewed data of 29 manuscripts available on linear and continuous B-cell immunogenic peptides of DENV1-4, ZIKV, JEV, WNV, and TBEV in infected humans. In total, 153 linear peptides were found in literature, whereas the majority of peptides was located in the E protein (68/153) followed by the NS1 protein (49/153) (69).

The E protein as a major surface protein has been proven to be targeted by cross-reactive Abs as shown in different enzyme-linked immunosorbent assays, tested with human monoclonal antibody (hmAb) panels or polyclonal human sera from ZIKV- and DENV-infected patients (9,47–49).

An important step in flavivirus diagnostic was taken by implementing ELISAs based on recombinant proteins, such as the E protein or the NS1 protein.

These antigens have shown to be more precise compared to using purified viral particles, but still mainly face low sensitivity (11–13,15,16,34,35).

Multiple algorithms based on machine learning or other computational epitope selection tools in bioinformatics have already been applied on prediction of epitopes and Ab binding from their structure or sequence especially in linear epitopes (70–72). Prediction methods for the discovery of flavivirus-specific discontinuous epitopes on the other hand are more complicated due to lack of spatial information or surface accessibility. Additionally, to further analyze putative, diagnostically relevant epitopes, new tools were invented which were based on inducing mutagenesis, for instance virus-like particle (VLP) antigen IgM antibody capture enzyme-linked immunosorbent assays (MAC-ELISAs) (73,74). A phage library is used to screen large libraries of proteins and detects especially conformational epitopes by extracting epitope-targeting Abs from serum (75–77).

Another approach was to identify a flavivirus-specific linear B-cell signature by using ZIKV and DENV peptides of prM, E or NS1 viral proteins in a peptide-based ELISA (78). In addition to this, a bead-based multiplex immunoassay (79) or a multiplex microsphere immunoassay (MIA) using recombinant viral antigens were implemented (80,81).

All in all, cross-reactivity especially in DENV and ZIKV occurs in serological assays (82–84) mainly for E protein (9,67,85,86) and NS1 protein (12,13,16,25,61,80,81), but is also found in NS2B (87) and NS5 (88,89), further complicated in secondary flavivirus infections (14,25,47,49,52,61).

1.3 Zika virus

The Zika virus was initially isolated in a serum of a rhesus monkey in the Zika forest, Uganda in 1947 (90). The increases in Guillain Barré syndrome (GBS) and fetal microcephaly following outbreaks in Southern America in 2015-2016 (91) urged the WHO to declare ZIKV a public health emergency of international concern in February 2016 (92). Three lineages of ZIKV exist, namely the East African, West African and Asian, which have very close phylogenetic relationship (44).

1.3.1 Humoral response and diagnostics

During natural course of ZIKV infection, major Ab targets are the preMembrane (prM) and envelope (E) proteins and nonstructural protein 1 (NS1) (49).

According to current guidelines, the diagnostic regimen of acute Zika or Dengue infection are very similar; the combination of a negative NAAT result and negative IgM Ab testing in serum specimens collected <7 days after onset of symptoms makes an infection rather unlikely (66). In symptomatic patients with possible contact to DENV and ZIKV, a positive NAAT result typically confirms acute infection. However, a negative acute IgM Ab test without NAAT testing can't reliably exclude flavivirus infection as Ab kinetics can vary in individuals and is time dependent [Figure 4] (66).

Indeed, when DENV or ZIKV IgM Abs are detected, but a NAAT or NS1 antigen test is negative, a confirmatory PRNT is indicated to ensure correct diagnosis. However, for the diagnosis of ZIKV after previous Dengue infection the PRNT is not reliable [Figure 4] (12,66). Besides the detection of IgM in serological assays described below according to U.S. guidelines, other assays including IgG or combined IgM and IgG Ab testing were introduced in recent years.

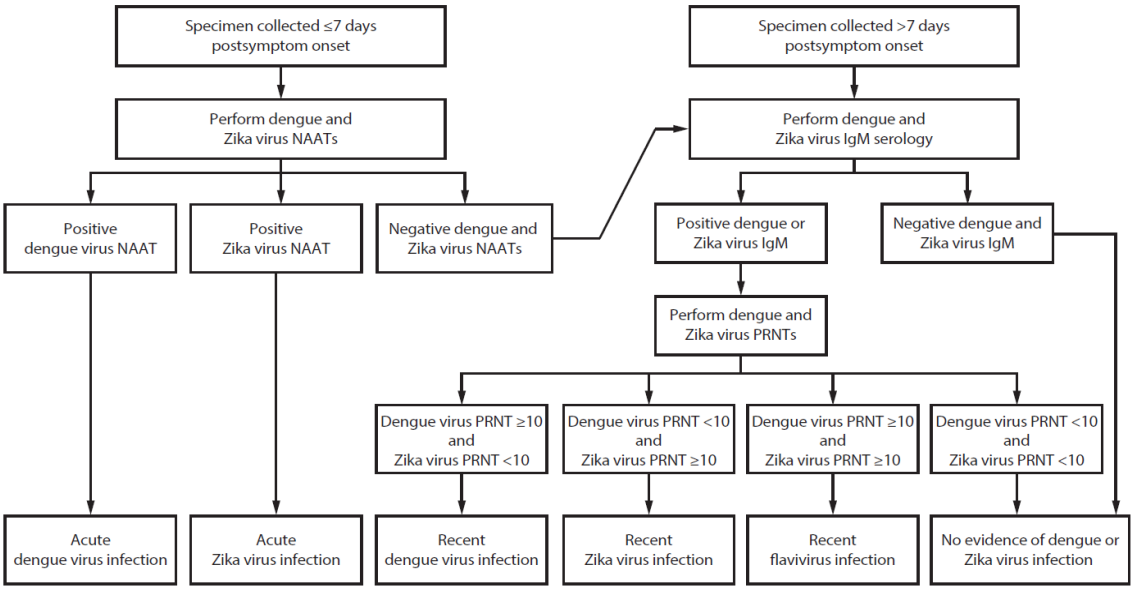


Figure 4 U.S. guidelines: Dengue and Zika virus diagnostics. Dengue virus nonstructural protein-1 testing can also be done instead of Dengue virus NAAT. Besides, IgG based serological assays, or the combination of IgM and IgG ELISAs can be considered. NAAT: nucleic acid amplification test; PRNT: plaque reduction neutralization test. Figure taken from (66).

One of the main commercially available and accurate tests in clinical diagnostics in Germany is the Euroimmun AG, Lübeck, Germany anti-ZIKV ELISA IgM and IgG NS1-assay (93), which has also been used in the serological analysis of ZIKV and DENV-infected study subjects used in this study. High specificity was reported for the Euroimmun ZIKV IgG ELISA assay in a cohort with samples of TBEV infection, TBEV vaccination, DENV infection, YFV vaccination and Hepatitis C virus infection (15). Taken the studies on performance of the Euroimmun NS1-ELISA together, the combined interpretation of results from both IgM and IgG ELISAs increased both sensitivity and specificity, while IgM or IgG alone showed poor performance (11–13,15,25,38). Higher specificity compared to Euroimmun assay was achieved in a ZIKV ED3 ICB ELISA using the ED3 antigen of the ZIKV E protein (54).

Limited cross-reactivity was shown with highly reactive recombinant NS1 proteins in a multiplex protein IgM and IgG microarray (52), but also in a recombinant NS1 BOB-ELISA developed by Balmaseda et al. (61) and further evaluated in a following study (14). Similar BOB-ELISA studies for detection of Dengue virus NS1 are still missing.

Previous studies which aimed for detecting serological markers for differentiation of ZIKV and DENV infections found flavivirus type-specific anti-NS1 Abs dominating the serological response in primary ZIKV infections (49) which were applied in a NS1 BOB assay and tested in a large multicenter study (61). Other studies were conducted for the detection of abs elicted after ZIKV infection using peptide-based ELISAs (94,95).

1.4 Dengue virus

The Dengue virus is one of the most important arthropod-borne viral infection in the world and was first isolated in Japan 1943. It is estimated that annually ~ 400 million cases of DENV infections occur and the global incidence of Dengue infections has grown dramatically in recent years (96). The DENV serocomplex comprises four closely related serotypes (DENV I, II, III, IV), which differ by 30–35% in amino acid identity. In particular, E proteins from different DENV serotypes had sequence identities of a minimum of 63% (44).

1.4.1 Humoral response and diagnostics

An overview of the stages of infection gives Figure 5. Compared to primary infection, the IgG response in secondary DENV infection already raises promptly during the first few days after onset of symptoms and is involved in ADE mechanisms.

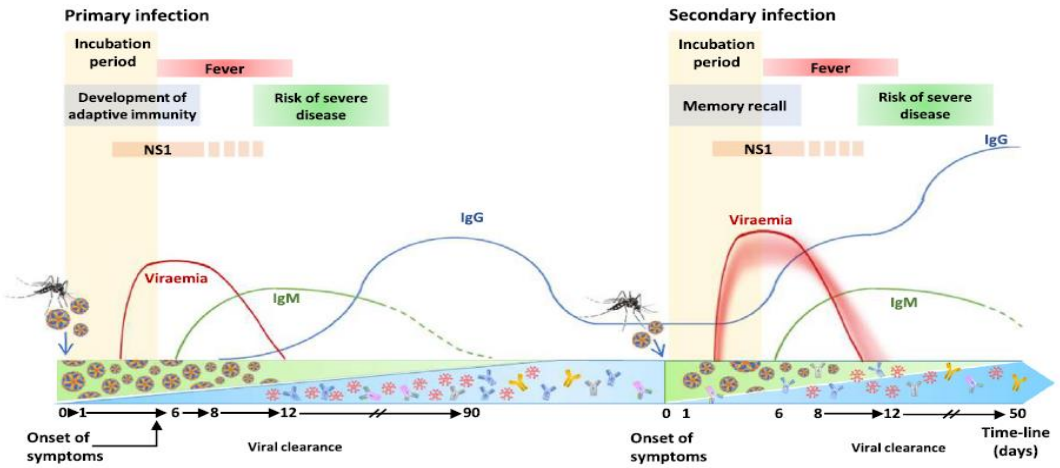


Figure 5 Timeline of infection in Dengue for primary (left) and secondary (right) infection. Figure taken from (64).

The diagnostics of ZIKV and DENV infections are described in Figure 4. In general, E and prM, and the non-structural protein NS1 have been identified as the main antigenic targets for human B-cell responses in DENV infections (28,49,82). In particular, the main Ab response to primary and secondary infection is directed against E protein (36,47,48,97,98). However, the serological response in DENV infection to the E protein showed a high serotype cross-reactivity (48), and correspondingly, most E protein-specific human mAbs detect and bind to more than one Dengue virus serotype (47). However, discrimination between DENV1 and DENV2 convalescent patient sera and convalescent DENV3 patient sera was possible in ELISA (99). In a peptide array spanning the proteomes of DENV-1, DENV-2, DENV-3, DENV-4, ZIKV, YFV and CHIKV performed with primarily DENV2-infected individuals, most immunodominant peptides were mapped to proteins C, prM, E and NS1 (100) concordant to previous results (45). Additionally, some reaction has been detected against prM/M protein (78,94,101–104). In primary and secondary DENV infection, NS1 antigen can be detected from onset of symptoms until up to 9 days (7,58,60,105).

NS1 ELISA and rapid diagnostic tests (RDTs) have proven to be generally more sensitive in acute phase specimens and in primary DENV infection, whereas IgM anti-DENV ELISAs and RDTs were less sensitive in secondary DENV infections due to NS1 cross-reacting Abs (2,11). This is correspondent to other findings in Western Blot analysis, where Ab responses to prM and NS1 proteins (48) and NS1 protein alone (106) were higher in patients with secondary infection than in primary infection.

The development of NS1 detection assays in acute phase and primary DENV infections (11,37,60,62,63,71) as well as in combination with standard diagnostics anti-DENV IgM ELISAs (107) or ZIKV IgG ELISA (108) have been widely implemented.

Ab responses have also been detected to other non-structural proteins like NS3, NS4 and NS5 – but Ab titers are low, especially in primary Dengue virus infections by Western blotting (106) and enzyme immunoassay (27). Nevertheless, a study of 2019 identified one DENV serotype specific epitope in the NS4B protein which was able to distinguish from Zika virus positive sera (109). Additionally, a superior assay performance was achieved for DENV-2 NS5 ELISA (110).

1.5 Yellow fever virus

The name flavivirus is derived from the first discovered virus of this genus, the yellow fever virus (flavus is Latin for yellow and jaundice is induced by YFV). The first clinical cases of yellow fever were described in Mexico in 1648 and is now widely spread over Africa and Central and South America (111).

According to phylogenetic studies including nucleotide sequence analysis, YFV is quite genetically distant from other flaviviruses and it is the most distantly related agent among mosquito-borne flaviviruses (112).

1.5.1 Humoral response and diagnostics

The diagnosis of YFV is concordant to diagnostics in DENV and ZIKV infections [Figure 4] with minor differences (113). An adequate performance in serological tests for both IgM and IgG detection in wild-type YFV infection is achieved in various laboratories worldwide (114). However non-specificity among flaviviruses particularly in DENV-immune sera has been observed (17,21).

It has been shown that false-positive cross-reactions with other flaviviruses appear rather in anti-YFV IgG based assays, although it also occurred in anti-YFV IgM and even neutralization testing (8,17,21,115).

Although many recent studies investigated the use of NS1 antigen as a biomarker in diagnosis of DENV infections, only a low number is available for YFV diagnostics: e.g. a quantitative NS1-capture enzyme-linked immunosorbent assay (116) or polyclonal peptide antisera for detection of YFV-C and -NS1 proteins (117). Furthermore, a RDT kit was developed to detect yellow fever virus (YFV) non-structural protein 1 (NS1) by mAbs (118).

1.5.2 Yellow fever vaccine

In 1937, Max Theiler developed an attenuated vaccine derived from YFV strain 17-D and was awarded the Nobel prize in Medicine in 1951 (119). Three sub strains of the 17D vaccine virus are produced in embryonated chicken eggs, namely 17D-204, 17D-213 and 17DD. Interestingly, the 17D vaccine is effective against all seven genotypes of wild-type YFV.

The parental Asibi strain and the derived YF-17D vaccine strain differ in 0.63% of nucleotide sequence scattered throughout the genome. In comparison with all other proteins, the envelope protein has one of the highest rates of amino acid changes, which is believed to play an important role in immunogenicity and attenuation (120,121).

Viremia as well as neutralizing IgM Abs are detected between three to seven days after inoculation of the vaccine and overall neutralizing Ab response is usually detected by seven days post vaccination (120). Vaccinees developed a strong and protective humoral immune response by PRNT on day 28 post YFV vaccination (68). The restricted replication and attenuation of 17D vaccine results in lower levels of viremia and specific Ab titers than in parental wild-type virus.

Further serological assays were developed and evaluated which still face difficulties to distinguish between Abs raised after natural YFV infection and YFV vaccination (114,122).

Results are reminiscent of data obtained by Niedrig et al. (19), where neutralization assay was proven the most sensitive test and the only one showing good correlation with YFV vaccination status when compared to YFV HI, YFV IFT and YFV IgG ELISA.

YFV vaccination sera showed low degree of cross-reactivity compared to other flavivirus infections or vaccinations in different immunoassays (18), in ELISA and HI assays (23,123), in ZIKV IgG ELISA by Euroimmun (10) as well as in IgM MAC-ELISA based on DENV antigen (21).

1.6 Tick-borne encephalitis virus

The TBEV is not endemic in the tropics/subtropics but occurs mostly all over Europe as well as Central and Eastern Asia. It is known as the most important arbovirus infection in Europe. Three different strains can be differentiated with close molecular identity: the European, Siberian, and Far Eastern strain.

1.6.1 Humoral response and diagnostics

Concordant to other flavivirus diagnostics the acute infection (first, viremic phase) is diagnosed via detection of viral RNA via PCR test complemented by the IgM capture ELISA with recombinant virus-like particles or purified virions (4,83). As in other flavivirus infection a positive test result of a commercially available TBEV ELISA can be undertaken by neutralization tests (83).

Additionally, the current commercially available IgM and IgG ELISAs face reduced specificity in TBEV infection, especially in IgG ELISAs (83,115,124,124–126). Further development was undertaken by the design of an IgG ELISA format with mutant envelope proteins of TBEV and WNV lacking cross-reactive, conserved regions of the fusion loop domain (127) or a recombinant NS1-based ELISA (128). Interesting results were collected in a study of Allwinn et al. (115) where the majority of healthy YFV vaccines were not positive by TBEV IgG ELISA. Secondly, no TBEV vaccinated study subject sample was positive by TBEV-HI assay, although all were positive by TBEV IgG ELISA.

1.6.2 Tick-borne encephalitis vaccine

In Europe two inactivated TBEV vaccines are available to date which are based on two European TBEV strains: Encepur® by Novartis Vaccines and Diagnostics, Germany, with strain K23 and FSME-IMMUN® by Baxter Bioscience, Austria, using strain Neudoerfl.

Besides the slightly different strains used in the two available vaccines, they differ in formulation as well. Occasional vaccine breakthroughs, cross-reactivity in serological assays across flaviviruses and (incomplete) TBEV vaccination history in patients complicate diagnosis in TBEV infection (129).

It was already found in 1985 that after administration of TBEV vaccination, individuals previously vaccinated with the 17D YFV vaccine developed TBEV IgG Abs in earlier stages and at higher titers with increased cross-reactivity to other flavivirus antigens in HI assay compared to previously non YFV vaccinated and flavivirus-naive patients (23). Furthermore, a study of 2019 showed impairment of neutralizing Ab response to TBEV vaccination in sera of previously YFV-vaccinated individuals (130).

The overall neutralizing Ab response was mainly directed against domain I and II (and/or the junction between them) of the E protein detected by various immunoassays using recombinant antigens of E and prM (20). Thus, only low virion reactivity and neutralizing activity were found in DIII depletion assay indicating a low significance for immune responses in TBEV infection similar to other results with YFV post vaccination sera (18). Interestingly, Ab detection against prM in TBEV post infection sera was higher in ELISA and depletion assays than in TBEV post vaccination samples. Additionally, broadly cross-reactive Abs were observed mostly in postvaccination sera (20). Nevertheless, in absence of cross-reactive Abs, IgG ELISA has shown to be a reliable method to screen for neutralizing Abs following TBEV vaccination (22).

Besides, the strong immunogenicity of the envelope protein, domain III (ED3) in particular induced specific, neutralizing Abs directed to the respective ED3 domain in WNV and DENV detection assays (34,35,55,56,97). The high specificity of EDE3 for detection of anti-TBEV Abs after infection as well as after vaccination was also shown in an immune complex (IC) ELISA when compared to two indirect ELISAs (131).

Ab responses to NS1 of TBEV are detectable post TBEV infection but are suggested not to appear and/or appear to low extent after vaccination as shown in IgM and IgG suspension multiplex immunoassay (SMIA) (132) as well as in immunoassay based on recombinant TBE NS1 (133) and in IgG NS1-based ELISA (134). Nevertheless, the specificity was low in individuals vaccinated against both YFV and TBEV (134).

1.7 West Nile virus

West Nile virus (WNV) was first recognized in the West Nile District of Uganda in 1937. WNV is endemic in large parts of the world, but is also geographically spreading throughout Europe within the last decade (135).

1.7.1 Humoral response and diagnostics

Concordant to other flavivirus infections, virus isolation or more frequently RT-PCR is used in first, viremic stage of infection. Diagnosis of WNV infection is clinically achieved by serological assays like HI assay, IgM and IgG in ELISA and the PRNT as the gold standard of flavivirus infection. The antigenic targets of immune response after WNV infection identified so far are predominantly located in the E, prM and NS1 proteins in either ELISA or IFAs. However, a microsphere immunoassay that identified Abs to NS3 and NS5 has also been described (89). Epitope blocking ELISAs are also available, which still face major issues in diagnosis of patients with history of previous flavivirus infection or vaccination (136).

The majority of mAbs developed after WNV infection are namely directed against domain II of the E protein (56) while the fusion peptide region is thought to be immunodominant but highly cross-reactive (56,137,138). The Ab response against prM in members of the JEV serocomplex has been known for a long time (56,102). While Cardosa et al. (102) detected prM Ab specificity in differential diagnosis in DENV and JEV infection, another study showed cross-reactivity of prM in WNV and SLEV via Western Blot. NS1 protein was declared a useful, specific marker for differentiation between WNV and SLEV (103). Additionally, the study of Johnson et al. (67) reported only a small degree of cross-reactivity in WNV infection in serologic assays after previous vaccination against YFV and JEV.

1.8 Research hypothesis and study objectives

We hypothesized that we could apply the peptide array technology for flavivirus type-specific peptide responses to map specific IgG responses after natural flavivirus infections and yellow fever vaccination to identify type-specific signatures of different flaviviruses. Cross-reactivity is suggested to be induced by Abs raised against conformational epitopes which might interact with additional quaternary and post translationally modified glycosylated epitopes or by other obscure mechanisms (51,139). This can make linear epitope detection via peptide arrays, where these epitopes and factors are not present, more suitable for invention of a diagnostic test. Besides its great potential, peptide array techniques haven't been implemented in clinical diagnostics of flaviviruses and remain an experimental tool.

In this doctoral thesis, the IgG response of peptide variants of specific proteins within flavivirus infections was studied in a high-throughput pan-flavivirus peptide array to:

- Compare performance of peptide array with plasma and serum samples
- Map specific IgG responses after natural flavivirus infections and yellow fever vaccination to identify type-specific signatures of different flaviviruses
- Study dynamics of cross-reactivity and cross-recognition between different flavivirus infections
- Compare antigenic regions targeted by IgG in TBEV-vaccinated and in TBEV-infected individuals

2 Materials & Methods

In total, we tested anonymized sera of 63 individuals after infection with DENV, ZIKV, TBEV, WNV, and YFV vaccination [Table 1] using peptide arrays. All samples were processed intermingling over the time period of laboratory work to prevent batch effects. The study was approved by the Ethics Committee of the Ludwig Maximilian University in Munich, Germany (project number: 19-713).

Subjects	Age (5 Year-Cluster)	Sex	Country of Origin/Ethnicity	Time Point of Sample Collection after Symptom Onset/ Vaccination (in Days)	Travel History	Flavivirus Vaccination History	Diagnostics
ZIKV (n=14)	15-65	m (n=6) f (n=8)	Germany, Austria, Switzerland, Peru	8-42	South America, Caribbean	n/a	DENV in-house PCR, ZIKV- and CHIKV PCR IFT IgM&IgG (for ZIKV; DENV; YFV; TBEV; WNV; JEV; CHIKV)
DENV (n=14)	15-65	m (n=8) f (n=6)	Germany	8-28	South America, South Asia, Caribbean	n/a	DENV in-house PCR , ZIKV- and CHIKV PCR IFT IgM&IgG (for ZIKV; DENV; YFV; TBEV; WNV; JEV; CHIKV) NS1 antigen assay and IgM & IgG NS1 ELISA
YFV-Vaccination (n=18)	15-45	m (n=6) f (n=12)	Germany	D0: before YFV vaccination D28: 28 Days after YFV vaccination	Before YFV vaccination: Europe, southern Africa, Oceania	TBEV-vaccinated (n=8) No flavivirus vaccination (n=10)	In house TBEV PRNT
TBEV (n=10)	n/a	n/a	Germany, Austria	n/a	n/a	n/a	IgM & IgG NS1 ELISA
WNV (n=7)	35-70	m (n=4) f (n=3)	Not Hispanic or Latino (n=6) Hispanic or Latino (n=1)	n/a	n/a	n/a	n/a

Table 1 Overview of study subjects. n/a: not available; m: male; f: female. More detailed table on study subjects see Figure S 1-S 4.

2.1 Subjects

2.1.1 Zika and Dengue virus

The 14 ZIKV and 14 DENV study subjects' samples originated from routine patient care in the outpatient clinic of the Department for Infectious Diseases and Tropical Medicine, University Hospital, LMU Munich. All study subjects gave informed consent for the use of their retained sera. Diagnoses had been secured by using a range of diagnostic tests [Table 1]. Serum samples were mainly selected based on certainty as well as characterization of infection and the magnitude of IgG titers for homologous infection (after seroconversion). All serum samples were stored frozen at –20°C until tested. Detailed tables of anonymized data characterizing each ZIKV and DENV sample can be found in the supplementary material [Figure S 1; S 2].

2.1.2 Yellow fever vaccination cohort

The yellow fever virus serum samples originated from a yellow fever vaccination cohort study using the vaccine Stamaril® (Sanofi) (principal investigator Simon Rothenfußer, Division of Clinical Pharmacology, University Hospital, LMU Munich). The study was approved by the responsible institutional review board of the Medical Faculty, LMU Munich. Sera from a total of 18 healthy participants prior vaccination (D0) and 28 days after vaccination (D28) were used. These 18 participants included a total of eight that reported a history of TBEV vaccination prior to inclusion in the yellow fever vaccination cohort. For all 18 sera, TBEV PRNT test results were available, but all eight sera of TBEV vaccinees had tested positive on D0 with titers ranging from 1:10 to 1:640 [Figure S 3]. All non-TBEV vaccinated study participants had zero neutralizing titers in TBEV PRNT on D28 of YFV vaccination and were therefore not tested in PRNT on D0. Histories concerning vaccination and/or infection with other flaviviruses were blank. Additionally, no participant reported a history of travel to the Americas or Asia and most participants haven't been travelling outside of Europe at all. Serum samples were collected between October 2015 and April 2016 and all samples were stored at −80 °C prior to use. A detailed table of data concerning each YFV serum sample can be found in the supplementary material [Figure S 3].

2.1.3 Tick-borne encephalitis virus

Ten study subject serum samples were obtained from patients after infection with TBEV from laboratories of the Bundeswehr Institute of Microbiology (IMB). Infections were presumably acquired in Germany or Austria. All samples were tested positive in a commercially available IgM and/or IgG NS1 ELISA shortly after symptom onset. The vaccination histories are incomplete, but study subjects partially had received TBEV vaccinations in the past. All samples were stored frozen at –20°C until tested.

2.1.4 West Nile virus

Seven WNV study subject samples originated from Vanderbilt Vaccine Center Biorepository. Convalescent sera were collected from individuals with confirmed previous history of natural WNV infection infected during an outbreak in Dallas, TX, U.S. in 2012.

The sera were drawn in the convalescent phase, months to years after post-onset of symptoms and have already been used in another study (140). A table of data concerning each study subject can be found in the supplementary material [Figure S 4].

2.1.5 Plasma vs. serum

In a pretest we analyzed plasma of two participants (D0 and D28) of the yellow fever vaccination cohort and compared it to serum sample results of the same two participants [Figure 10].

Besides the samples mentioned in Table 1, repetitive testing of serum from one female YFV participant at day 28 (D28) after YFV vaccination was performed each month for quality control to test inter-assay variability of the peptide array; see supplementary material [Figure S 5].

2.2 RepliTope™ pan-Flavivirus peptide microarray

The peptide array method was used in this thesis to identify flavivirus-specific antigens recognized by IgG. In these assays, an amino acid sequence representing a short synthetic peptide is synthesized, chemically immobilized, and then spotted on a solid surface, usually a microscopic glass slide or a microchip by in-situ synthesis or rather SPOT-synthesis.

It is used to specifically detect Abs (mostly IgM and/or IgG) that target these antigen-derived purified synthetic peptides by using a secondary antibody, whose fluorescence signals are read out by a Laser scan. Nowadays, it enables high through-output screening of a various number of (un)known peptides from different viruses or even the whole proteome with high-density peptide arrays. These short amino sequences forming peptides display B-cell epitopes, which lie closely together on the protein surface and determine antigenicity. In general, the assay technique is similar to an ELISA test. It can be incubated with a variety of different biological samples; however, in our study we exclusively used human sera.

Peptide microarrays enable to specifically dissect (in our study IgG) monoclonal or even polyclonal Ab responses from other heterogenous immune responses and further analyze the epitope-binding measuring intensity of fluorescence signals.

In addition, besides the qualitative and quantitative analysis it opens doors to parallelly study Ab responses to diverse antigenic regions and variants. Then, synthetic peptides are more stable on a chemical and physical level than for example proteins and lead to very steady method framework (141).

The advantages of peptide microarrays compared to commercially available diagnostic tools like ELISA, or protein-based microarray are multifaceted: Besides the simultaneously and efficiently detection of Ab responses against a high number of peptides, it creates signal intensities correlated with certain levels of Ab amounts in comparison to standard ELISA-based Ab detection.

Besides, it constitutes remarkable advantages in terms of specificity and costs: Peptide microarrays require only an amount of µL per sample and enables fast, quantifiable fluorescent readout and automated computational analysis with easy-to-use software to generate heat maps and identify binding regions. So we expect to tell even slight changes in the whole antibodyome (141). In comparison with proteins in a protein-based array, peptides are easily produced, chemically stable, and compatible with different immobilization chemistries. Additionally, peptides can partly conserve protein function (142).

The potential of using a peptide microarray tool is highly attractive, and multiple diagnostical approaches in various fields of medical research were conducted to date, for instance to study the inhibition and functionality of enzymes, ligands for cell behavior or to map autoepitopes and allergen epitopes. The possibility of synthesizing a genome-wide peptide microarray and successfully perform comparative epitope modeling has been used in others than flavivirus infections in humans (143–148), in vaccination (149) as well as in flavivirus diagnostics before (3,62,71,87,140,150–154).

The RepliTope™ pan-Flavivirus peptide array used in this study consists of a glass slide spanning an area of (2.5 x 7.5cm). On each glass slide, an antigen library of 3x6253 immobilized peptides with a peptide length of 15 AA were printed via SPOT-synthesis and immobilized on the array, see Figure 6. As each peptide is deposited in triplicate, the peptide array can be divided in three individual subarrays.

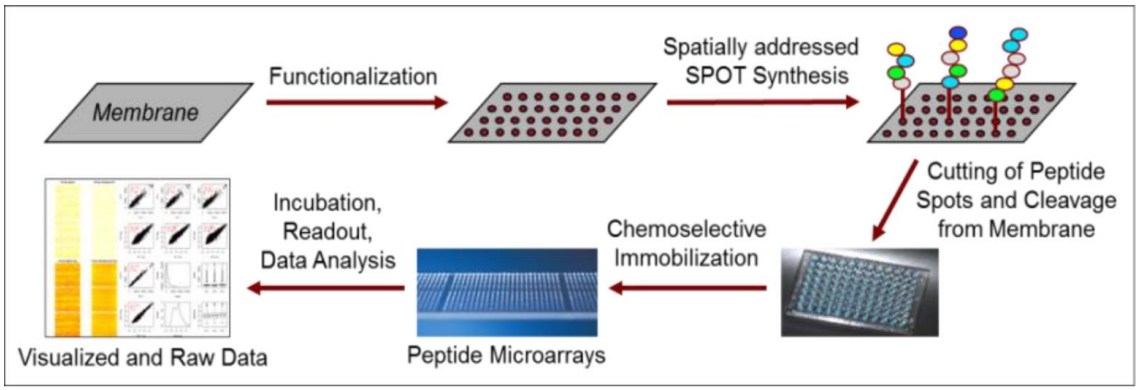


Figure 6 Production of peptide microarray used in this thesis; figure provided by JPT Peptide Technologies GmbH.

The purified synthetic peptides displayed on our peptide array are derived from antigens of DENV I-IV, YFV, ZIKV, SLEV, WNV. The amino acid sequences for every virus were selected from the NCBI-Protein database in February 2016. The sequences from the different isolates differ, which made an algorithmic preselection of protein sequences necessary to ensure optimal coverage (as shown in percent). For YFV, ZIKV, SLEV and WNV, two sequence groups were identified according to phylogenetic alignments for each virus/serotype.

Consensus sequences for each group were calculated and sequences with highest similarity to the consensus or rather representative strains selected for library design, see Table 2.

		Capsid protein C	Peptide pr	Small envelope protein M	Envelope protein E	Non-structural protein 1	Non-structural protein 2A	Serine protease NS3; Non-structural protein NS3	RNA-directed RNA polymerase NS5
Antigens	**#isolates**	**C**	**pr**	**M**	**E**	**NS1**	**NS2A**	**NS3**	**NS5**
Dengue_I	1195	82.96	84.74	85.18	90.06	92.4	79.38	94.14	94.52
Dengue_II	922	81.47	74.18	74.82	90.22	88.7	76.79	91.35	91.3
Dengue_III	677	88.54	87.91	90.1	92.6	93.1	91.36	96.78	94.23
Dengue_IV	140	87.96	90.35	86.67	93.19	86.75	78.35	95.78	91.55
YFV	58	95.32	96.81	97.92	94.53	96.11	88.9	97.34	94.85
ZIKV	38	95.87	96.7	95.17	98.2	98.75	97.18	99.2	98.12
SLEV	34	96.11	94.25	98.82	97.62	95.7	96.78	96.71	96.91
WNV	950	98.7	98.34	98.59	98.89	97.33	98.29	99.37	99.2

Table 2 Number of isolates and percent coverage of residues for all flavivirus antigens and proteins according to representative sequences; figure based on data provided by JPT Peptide Technologies GmbH.

Additionally, Capsid protein C, p62, envelope glycoprotein E1, E2 and E3 as well as 6K protein of Chikungunya virus were included on the array as a control to detect possible non-specific reactions to a clinically important, more distant genus of alphavirus. The glycoprotein E2 has been shown to play an unique role inducing a specific anti-CHIKV Ab response and can therefore potentially serve as a serological detection marker for CHIKV infection (64,155).

The Batch 3207 of the RepliTope™ pan-Flavivirus peptide microarray was obtained from the company JPT Peptide Technologies GmbH, Berlin.

2.3 Laboratory experiment and peptide array read-out

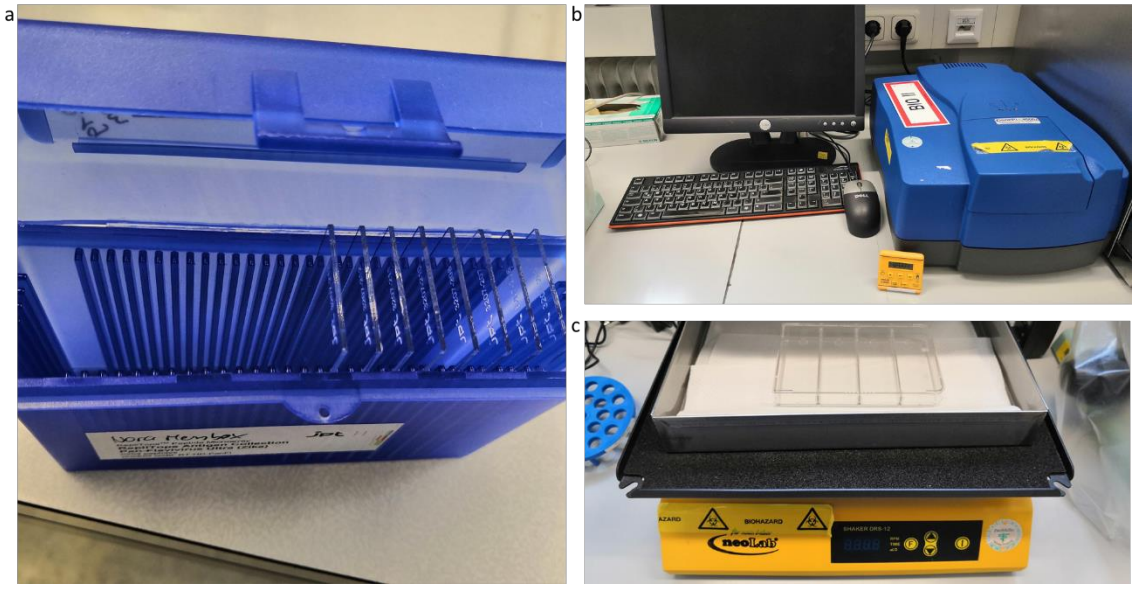


Figure 7 Laboratory setup and equipment. a) box with peptide array slides b) Computer with installed GenePix Pro 6.0 software and blue GenePix 4000A scanner. c) aluminum box on rocking platform/shaker. Inside lays the transparent incubation trough with peptide array slides in each of the four chambers.

All sera underwent one freeze-thaw cycle prior to use to ensure that each subject's serum sample was processed under the same conditions. The pre- and postvaccination sera of the yellow fever cohort from each individual were processed simultaneously. The microarray slides were stored at 4°C (not frozen). During the laboratory experiment, the peptide microarray slide surface was never touched with pipette-tips or fingers and all pipetting was executed on the edge of the chamber. All working steps were carried out under the hood; an overview of the laboratory setup is given in Figure 7.

The dilution of the 2nd fluorochrome-labelled antibody at 1:20000 was pretested with standardized HIV positive serum on an HIV Envelope-spanning peptide array provided by JPT prior start of laboratory work to ensure optimal balance between serum dilution and assay sensitivity.

The Microarrays were processed according to the manufacturer's instructions (JPT Peptide Technologies) with minor changes as described before (156) [Figure 8].

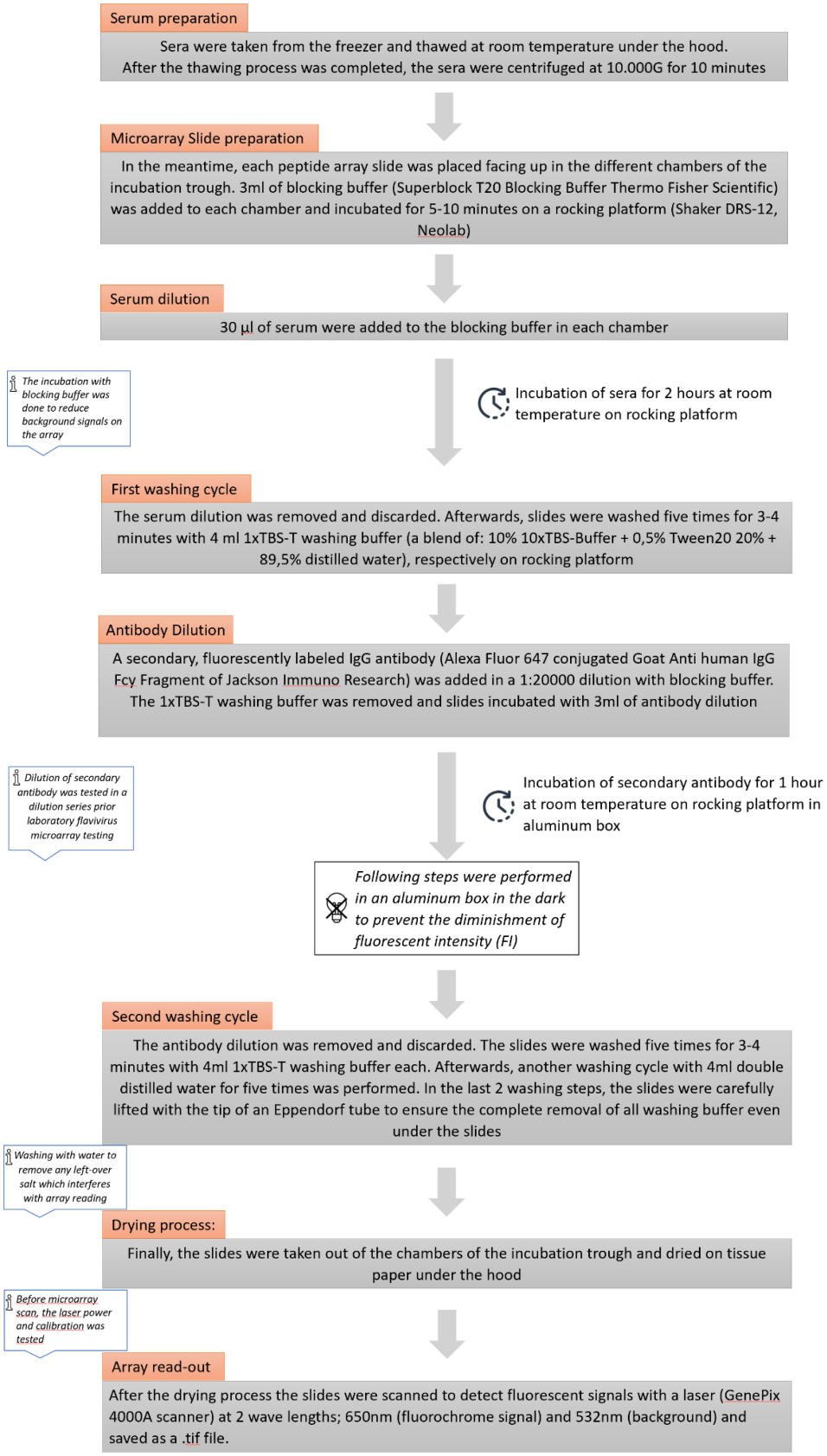
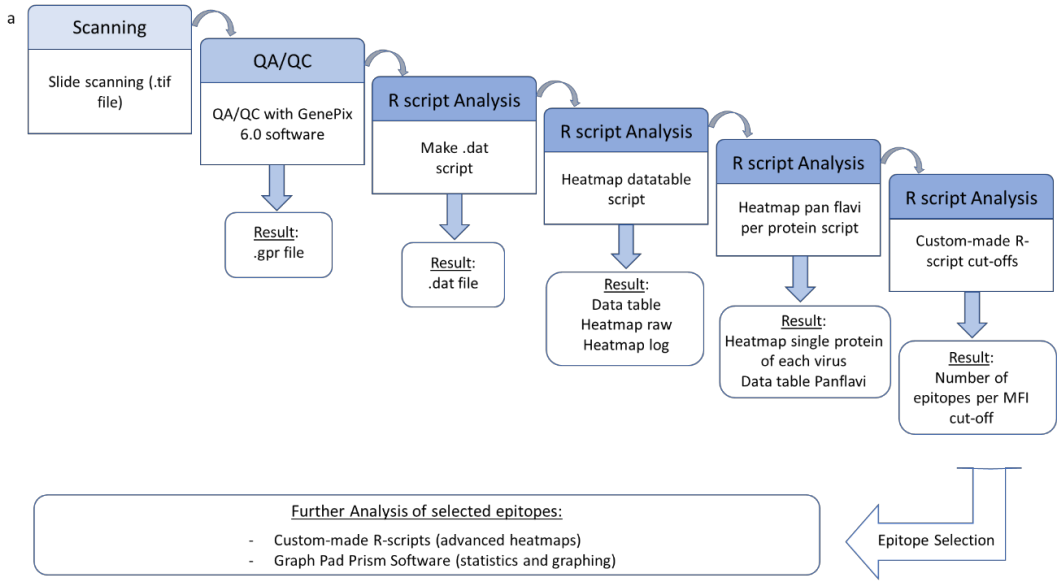


Figure 8 Processing of peptide array slides in the laboratory.

An overview of the following data analysis of the peptide arrays is shown in Figure 9a. After processing the slides in the laboratory and the generation of a .tif image file by the GenepixPro 6.0 software (Molecular Devices, San José, CA, USA), an array specific .gal file provided by JPT was processed with the GenePix Pro 6.0 software. The .gal files are tab-separated text files to complete the array layout for individual identification and location of each spot on the microarray surface. It contains a grid, which was overlaid on the resulting image to ensure the correct positioning of individual spots. Each spot in the microarray stands for a separate individual peptide. The software automatically flags features that fail background threshold criteria as “not found”. Manual adjustments were made to better capture the signal and to exclude artifacts caused by either dust, particles, fingerprints or else, which interfere with the final signal read out. The GenePix Pro 6.0 software can quantitate the fluorescence signal in each spot, calculate the red/green fluorescence ratio and uses log transformation for the ratios (157). The most important feature for the following analysis is the calculation of the mean for the signal intensity values regarding the existence of three identical spots per array subunit according to provided protocol by manufacturer JPT. The results of the QA/QC process were saved as a .gpr file [Figure 9b-e]. The same analysis was performed to compare performance of peptide array with plasma and serum samples to create heat maps and graphs [Figure 10].



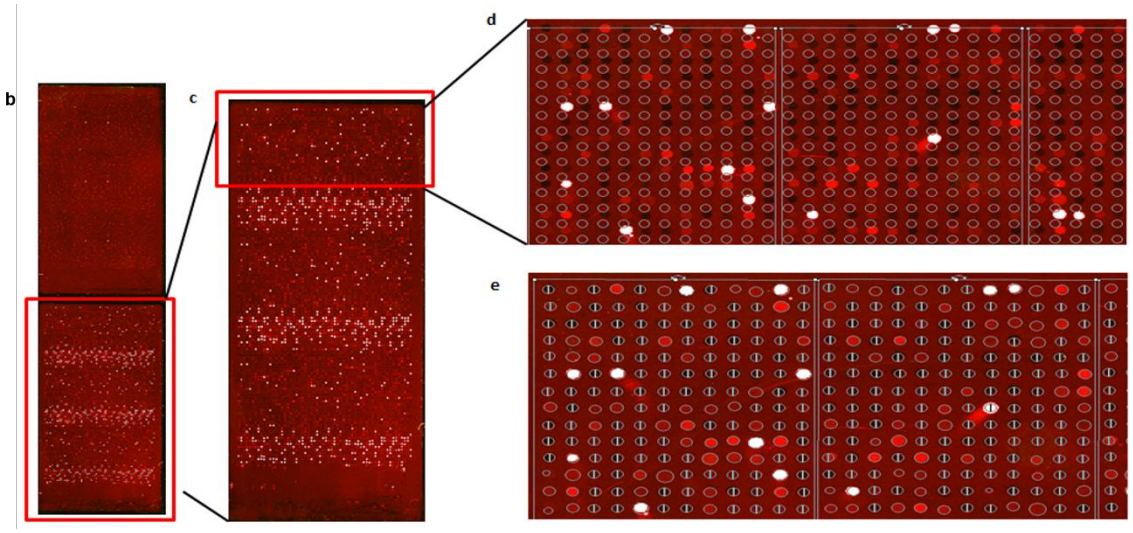


Figure 9 a) Overview data analysis (b-e) The generation of the .gpr file, b) the whole .tif file (= one slide) with peptide triplicates c) closer look at one subarray d) overlaying the "grid" on the .tif file, e) correct manual alignment of individual peptides/spots. Figure taken from (158).

2.4 Bioinformatic data analysis

The selection of proteins of different flaviviruses included in the following analysis is based on related work on peptide arrays or similar serological methods for identification of (linear) b-cell epitopes in flavivirus-specific immune responses.

The panflavi peptide array work of Viedma et al. (140), Keasey et al. (3) and Cleton et al. (62) found mainly immunodominant epitopes in NS1 and E protein of DENV, ZIKV, WNV, JEV, SLEV and YFV. For DENV specific epitopes within prM, E and NS1 proteins (71,78,150) and in ZIKV epitopes within prM, E, NS1 and NS3 (70,78,87,152–154,159–161) were considered promising for inducing a flavivirus-specific IgG response. For TBEV, Kuivanen et al. (151) identified epitopes in E and NS5 protein in a peptide array which reacted in majority of TBEV-infected study subjects and proved not to be cross-reactive to DENV1–4 or WNV IgG.

Subsequent analysis of data was conducted using open-source programming language R in an integrated development environment called RStudio (version 1.4.1106) and Microsoft Excel (162). The R-scripts (text file with commands) of the program were written and provided by Dr. Reimer from JPT and slightly modified by us. An overview of the analysis using R following the QA/QC process generating a .gpr file is displayed in Figure 9a.

We first applied a make.dat script to convert the .gpr file created by the GenePix Program after the QA/QC step into a .dat file, which mainly combines the mean fluorescence intensity values (MFI) or rather magnitude of peptide recognition for each peptide response from triplicate values. Precisely, opening this generic data file in Excel shows raw data of the triplicates SA1, SA2, SA3, the mean, median and standard deviation (SD) of fluorescence intensities.

In addition to this, the coefficient variation (CV; standard deviation divided by the mean) is calculated for each peptide based on the mean fluorescence intensity of pixels of the multiple signals to exclude a single signal outlier. Thus, when the CV is greater than 0.5 only the two measured values having the least difference (mean of the closest two, MC2) are used for the analysis of the final signal.

The MMC2-value present in the .dat file is the mean of the MC2 value (mean for all measured intensities without outlier). The data presented in the .dat file for each individual study sample is used for application of a following heatmap datatable R-script. The results are a first data table and two preliminary heat maps. In the data table, the MMC2 values for each sample for the total 6256 peptide sequences respectively, are listed in Excel format. The preliminary heat maps show the signal intensities of the serum samples (x-axis) depending on all different peptides (y-axis) [Figure 11]. A following heatmap pan flavi per protein R-script was run resulting in a graphical output as singular heat maps for different proteins of each flavivirus [Figure 12; Figure 13; Figure 14; Figures S 6-S 11]. The order of overlapping peptide sequences on y-axis in heat maps is from its N terminus toward its C terminus. A red bar displays a strong IgG epitope recognition of 100% MFI in the sample followed by descending color coding from red to light yellow to white meaning no IgG recognition. These heat maps are useful for visualization and identification of major binding sites.

A high, not explainable reactivity was detected in one YFV serum 005_Y at both timepoints D0 and D28 and it was therefore excluded from the YFV control group D0 for further analyses.

Besides, due to the overall unspecific low magnitude of peptide recognition as well as side-by-side random patterns of IgG recognition across both timepoints D0 and D28 in the YVF vaccination sera seen in heat maps [Figure 13; Figure S 7], analyses with yellow fever postvaccination sera (D28) weren't continued. The focus was hereafter placed on the ZIKV-, DENV-, WNV- and TBEV-infected study subjects.

Since TBEV antigens are not displayed on the array, the MFI values of TBEV-infected study subjects reacting against WNV and SLEV antigens were examined as seen representationally in the heat map of TBEV-infected and TBEV-vaccinated individuals reacting against WNV E protein [Figure 14].

Apart from the creation of singular heat maps for different proteins of each flavivirus, a data table panflavi was generated with the heatmap pan flavi per protein R-script [Figure 9a] which assigned each mean fluorescence intensity (MFI) value of the different serum samples to one corresponding epitope located in a protein of a virus. Some peptide sequences were similar between different viruses and were therefore expressed as duplicates or even triplicates. All in all, the table demonstrates the numeric output of the microarray analysis (table not shown). In the next step, all the mean fluorescence intensity (MFI) values for all epitopes of the proteins of interest within ZIKV, DENV, WNV, SLEV namely E, prM, NS1, NS3 and NS5, were automatically extracted from the table.

To focus the analysis on highly reactive epitopes, immunodominant regions (IDR) were then defined by a frequency of responders (FOR) of >50% at an individual, self-defined flavivirus cutoff value, which has been successfully applied before (156). Thus, a custom-made R-script cutoffs was written to define cut-offs at different MFI values (from 1000 to 12000 in steps of 500) [Figure 9a]. Whenever over 50% percent of study subject within a flavivirus type infection showed values above 6000 to 8000 (depending on the virus) for a given peptide/antigenic region, the highest value was chosen out of the data table and inserted in a new excel file resulting in an individual table for every protein/virus containing all MFI values for every study subject sample (excel table not shown). The selection of cut-offs represents a compromise between sensitivity and specificity.

The peptide-specific IgG responses or rather the number of epitopes that were found positive (frequency of responders (FOR) of >50%) at a selected cutoff threshold range between 3-52 per protein per virus of interest and are demonstrated in Figure 15.

To validate our hypothesis and to measure epitope specificity of previously selected epitopes, we juxtaposed MFI values of selected potential flavivirus-type-specific epitopes of each flavivirus-infected study subject in an advanced heat map with z-scores [Figure 16]. Z-scores represent the distance of a value from the mean of each row, which are MFI values for each peptide measured in standard deviations.

In detail, a subtraction of the mean from each peptide value is performed and then divided by the standard deviation along the row. The colors are computationally assigned with stronger colors having greater values, therefore have a higher z-score in standard deviations from the mean to display the variance in value across samples. The MFI values of the yellow fever vaccination cohort (YFV-D0) were added as controls. An additional advanced heat map was created for displaying MFI values of different flaviviruses against all selected epitopes by pairwise alignment of study subject sera according to z-score similarity demonstrated in Figure S 12. We also analyzed our data using lasso script and clustering, but it has proven to be unsuitable for our purposes (data not shown).

2.5 Statistical analysis

Besides visualization in form of heat maps by a custom-made R scripts, the magnitude of peptide recognition in form of MFI values were plotted on graphs using the software GraphPad Prism version 6.01 (163). The statistical significance of our results was determined by the nonparametric Mann–Whitney U test. The Mann-Whitney U test consist of the comparison of two unpaired groups, which in this case are the different virus groups or rather the MFI values obtained by the different flavivirus sera. Differences were considered statistically significant at $p < 0.05$.

3 Results

Our analyses using mainly R-scripts and GraphPad Prism software (163) resulting in color-coded heat maps and graphs . The results are exemplarily shown broken down by each flavivirus and protein in the following chapters.

3.1 Plasma vs. serum

Continuous IgG recognition patterns are demonstrated regardless of YFV vaccination plasma or serum samples. Comparable performance of peptide array across each study subject for timepoints D0 and D28 can be presumed with only minor MFI value differences in single epitopes of NS1 protein in plasma samples of study subject 009_Y at timepoint D0 and D28.

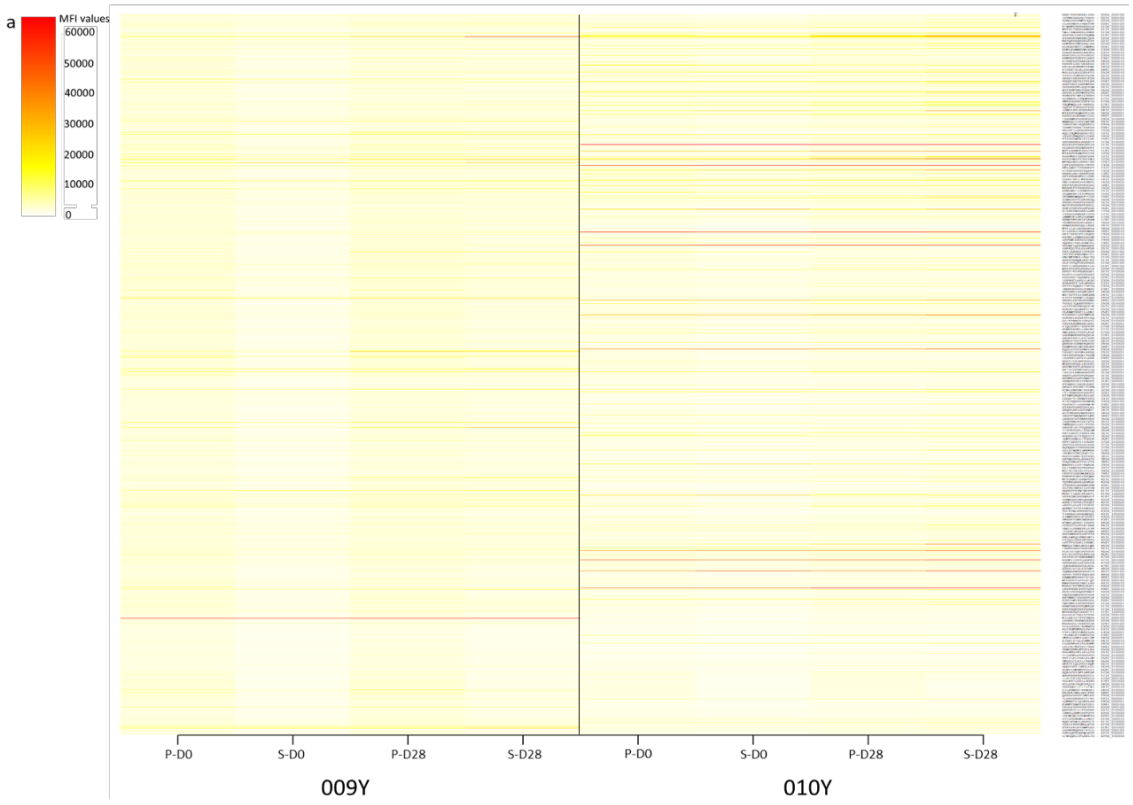


Figure 10 a)-c): Comparison of performance of peptide array with YFV vaccination cohort plasma and serum samples D0 and D28. a) Color-coded heat map of study subject 009_Y (left part of heat map) and study subject 010_Y (right part of heat map) with plasma (p) and serum (s) sample paired next to each other. X-axis: study subject sera; y-axis: all 6253 peptides along the sequence (N-terminus: bottom); legend to MFI values located on the left. b)-c): Further unraveling of IgG recognition in different epitopes on x-axis of YFV E protein (b) and YFV NS1 protein (c) which are plotted against mean fluorescence values (MFI) on y-axis. X-axis: different epitopes of protein; y-axis: MFI values.

3.2 Heat map analyses

3.2.1 Raw data

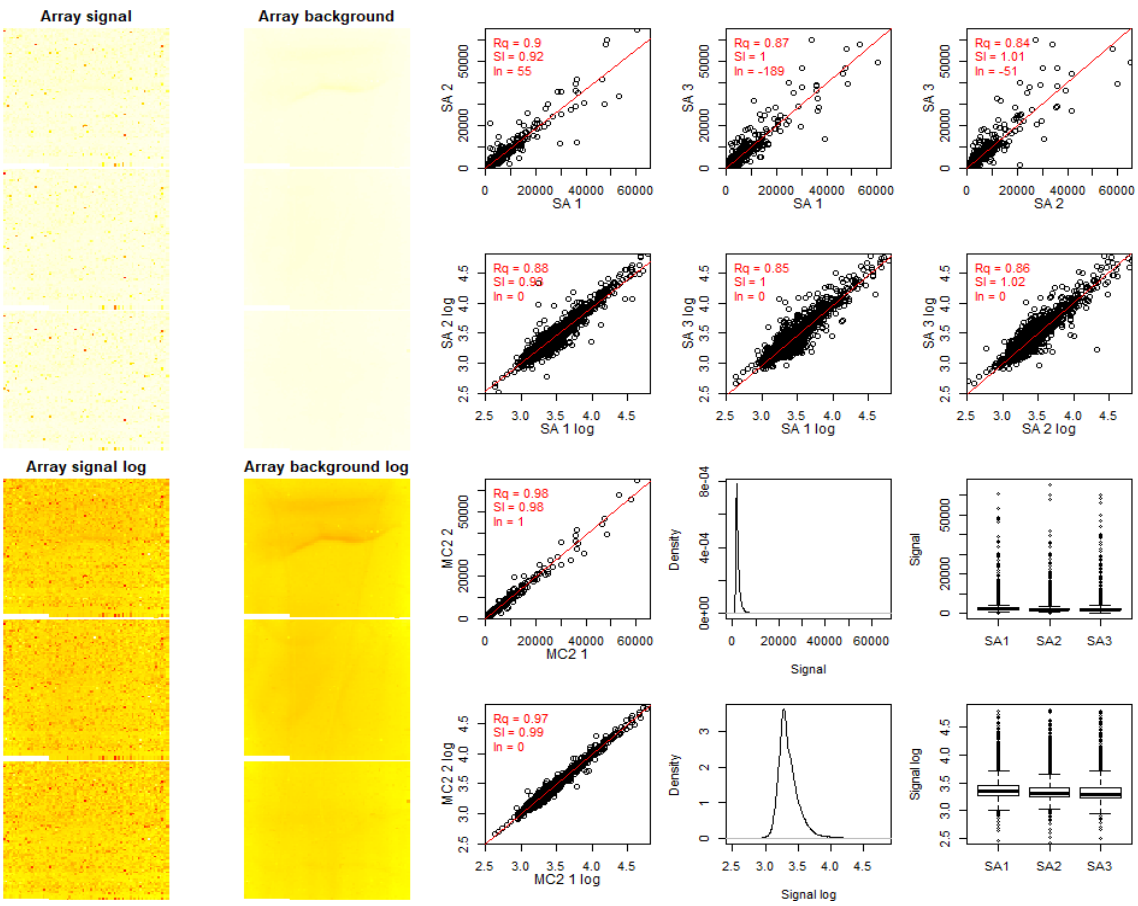


Figure 11 Preliminary heat map, here shown for study subject 009_Y at timepoint D0 Left side: picture of different array and background signals with and without a logarithmic scale. Background signals on the array are present to a certain degree and no background subtraction was performed. Right side: graphs of raw data of the triplicates SA1, SA2, SA3, as well as mean of the closest two (MC2) plotted against each other on the graphs with or without a logarithmic scale, further read out and visualized by following heat maps.

3.2.2 Heat maps of IgG peptide recognition for single proteins

In the following, representative heat maps for DENV E & NS1 protein [Figure 12] and YFV study subjects prior (D0) and 28 days after vaccination (D28) [Figure 13] are shown.

Heat maps for ZIKV E, prM, NS1 and NS3 in ZIKV-infected individuals; YFV NS1 protein in prior (D0) and post YVF vaccination (D28) sera; SLEV E in TBEV-infected vs. TBEV-vaccinated (YFV-D0) study subjects; WNV & SLEV E and WNV & SLEV NS5 in TBEV-infected individuals as well as WNV E, NS1, NS5 in WNV-infected study subjects underlining results discussed below can be found in the supplementary material [Figures S 6-S 11].

All in all, no continuous epitope recognition pattern (red bars) across all study subjects infected with ZIKV; DENV, TBEV and WNV can be seen. The IgG response in the YFV vaccination cohort is indistinguishable when unraveling the factors YFV vaccination (D0 vs. D28), TBEV-vaccination (Non TBEV-vaccinated vs. TBEV-vaccinated) and age [Figure 13; Figure S 7]. Additionally, high MFI values of 20000 to 60000 are already seen in flavivirus infection-naïve study subjects prior vaccination (D0). In Figure 14 a stronger IgG response in TBEV-infected individuals is elicited compared to vaccinated ones, however no specific region targeted by IgG in one of the groups compared to the other.

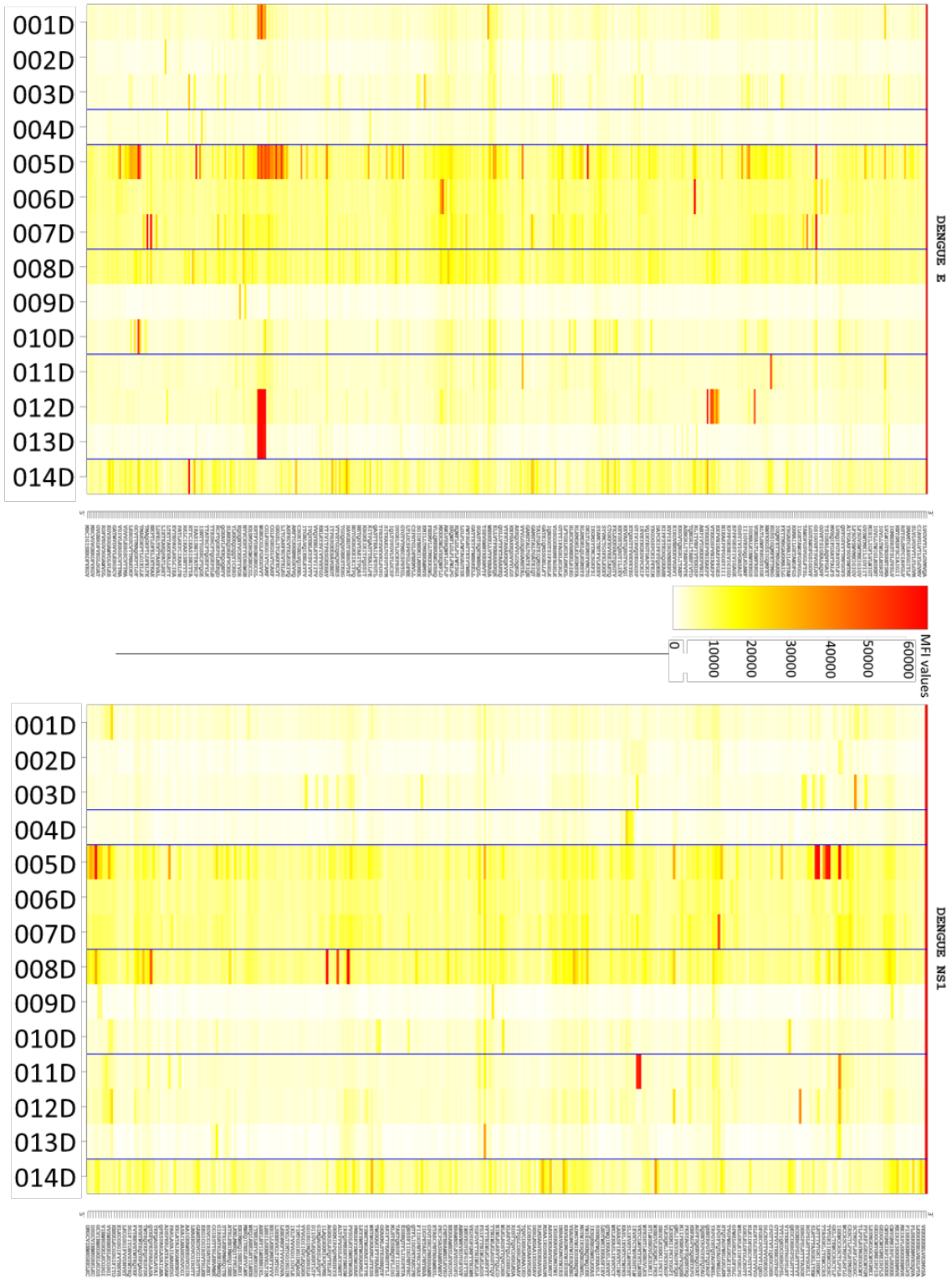


Figure 12 Color-coded heat map of DENV serum samples. Mean fluorescent intensities (MFI) of DENV sera on x-axis plotted against all different peptide sequences mimicking epitopes of the DENV E protein (upper heat map) and DENV NS1 protein (lower heat map) on y-axis. X-axis: study subject sera; y-axis: peptides along the sequence (N-terminus: bottom); legend to MFI values located in the center.

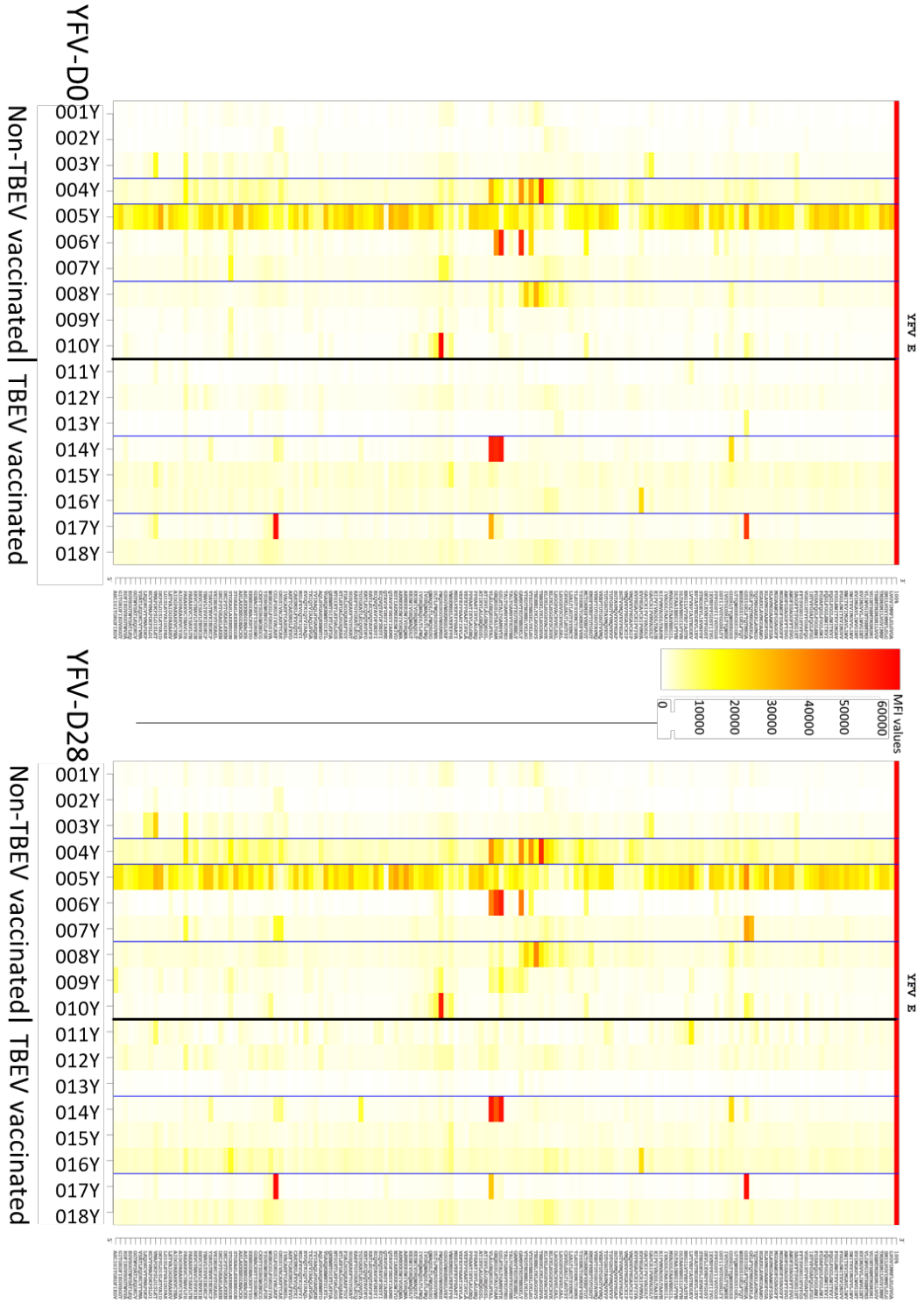


Figure 13 Color-coded heat map of YFV-vaccination serum samples. Samples prior (YFV-D0, upper heat map) served as our control group and samples after vaccination (YFV-D28, lower heat map) are displayed, subdivided into study subjects with no history of TBEV vaccination (non-TBEV-vaccinated) and with history of TBEV vaccination (TBEV-vaccinated). Mean fluorescent intensities (MFI) of YFV-vaccinated sera on x-axis plotted against all different YFV E protein peptide sequences on y-axis. A further subdivision is made by age, displaying the old generation (age 25-44) on the left of each heat map until study subject 005_Y in the non TBEV-vaccinated group and study subject 016_Y in the TBEV vaccinated group followed by the young generation (age 15-24), respectively. Related heat map for YFV NS1 protein is shown in Figure S 7 in the supplementary material. X-axis: study subject sera; y-axis: peptides along the sequence (N-terminus: bottom); legend to MFI values located in the center.

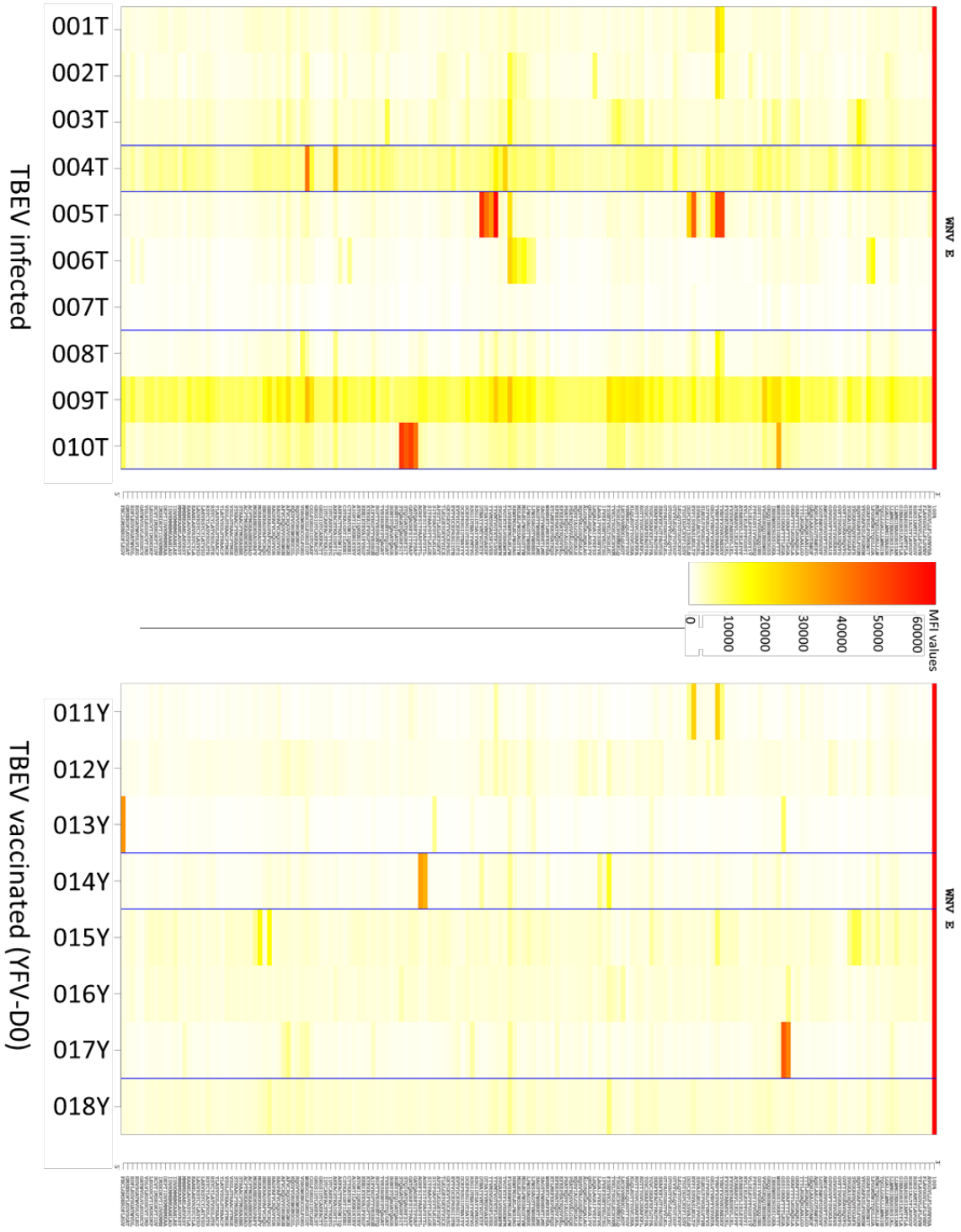


Figure 14 Color-coded heat map of TBEV-infection (upper heat map) and TBEV-vaccination serum samples (YFV-D0, lower heat map). Mean fluorescent intensities (MFI) of TBEV serum samples on x-axis plotted against all different WNV E protein peptide sequences on y-axis. TBEV antigens aren't included on the array, therefore IgG responses are demonstrated for related WNV. X-axis: study subject sera; y-axis: peptides along the sequence (N-terminus: bottom); legend to MFI values located in the center.

3.3 Epitope selection to define flavivirus type-specific IgG recognition patterns

For DENV, TBEV and WNV, the cut-offs were set between MFI values of 6000 to 6500. Therefore, the peptides selected for further analysis represent a very small proportion (<25%) of the total count of peptides displayed on the array. Figure 15 demonstrates that ZIKV-infected study subjects showed higher reactivity against ZIKV proteins and Dengue NS1 protein than other sera of flavivirus-infected study subjects against their autologous proteins. Therefore, the cut-off for selection of ZIKV peptides was set at MFI value of 8000, which results in roughly equal amounts of selected peptides. After the cut-off definition was performed, all peptides at the certain value were selected and provide the basis of the following analyses.

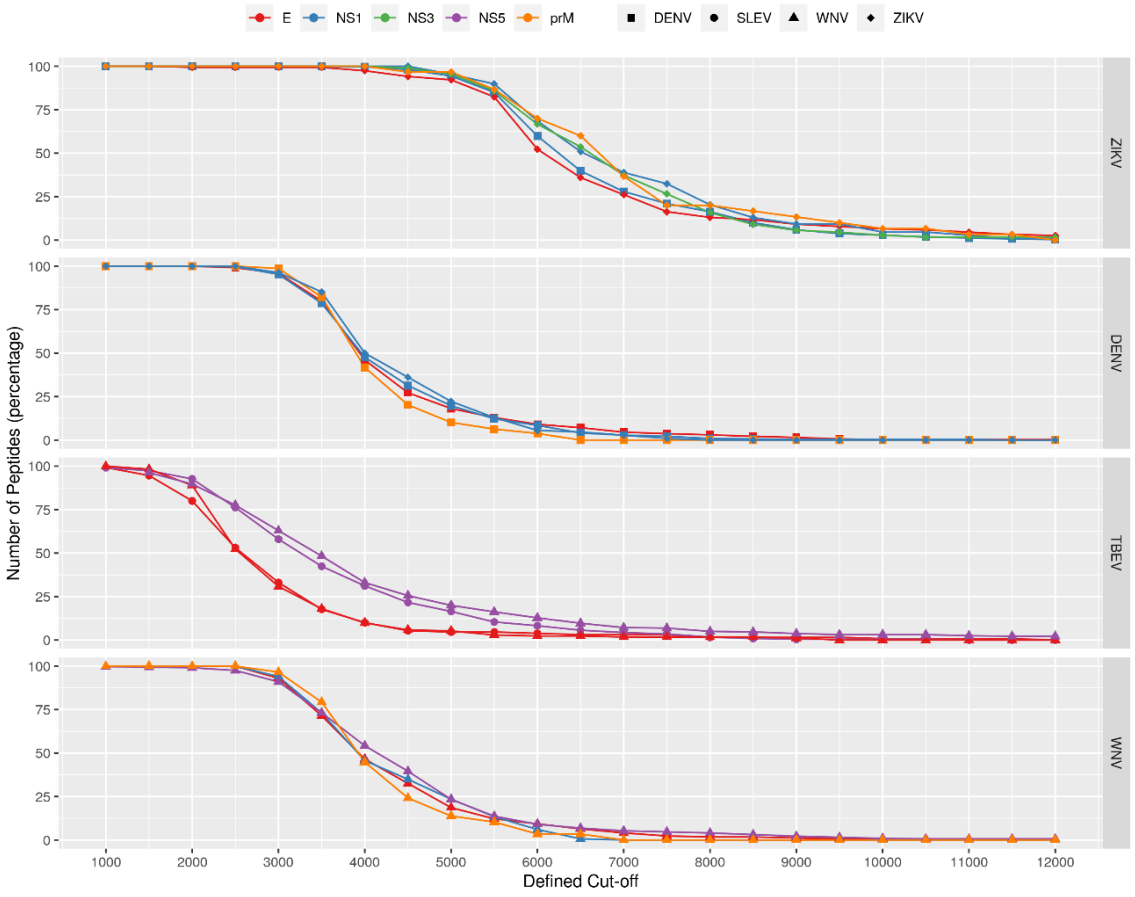


Figure 15 Cut-offs (MFI values) for epitope selection of immunodominant regions (IDR) defined by a frequency of responders (FOR) of >50% for each virus. TBEV is not displayed as an antigen on the array. Therefore, the IgG response of TBEV-infected study subjects against the two most related flaviviruses displayed on the array, namely WNV and SLEV antigens, were analyzed. X-axis: defined cut offs; y-axis: left: number of peptides in percentage; right legend: different flaviviruses.

3.4 Advanced heat maps

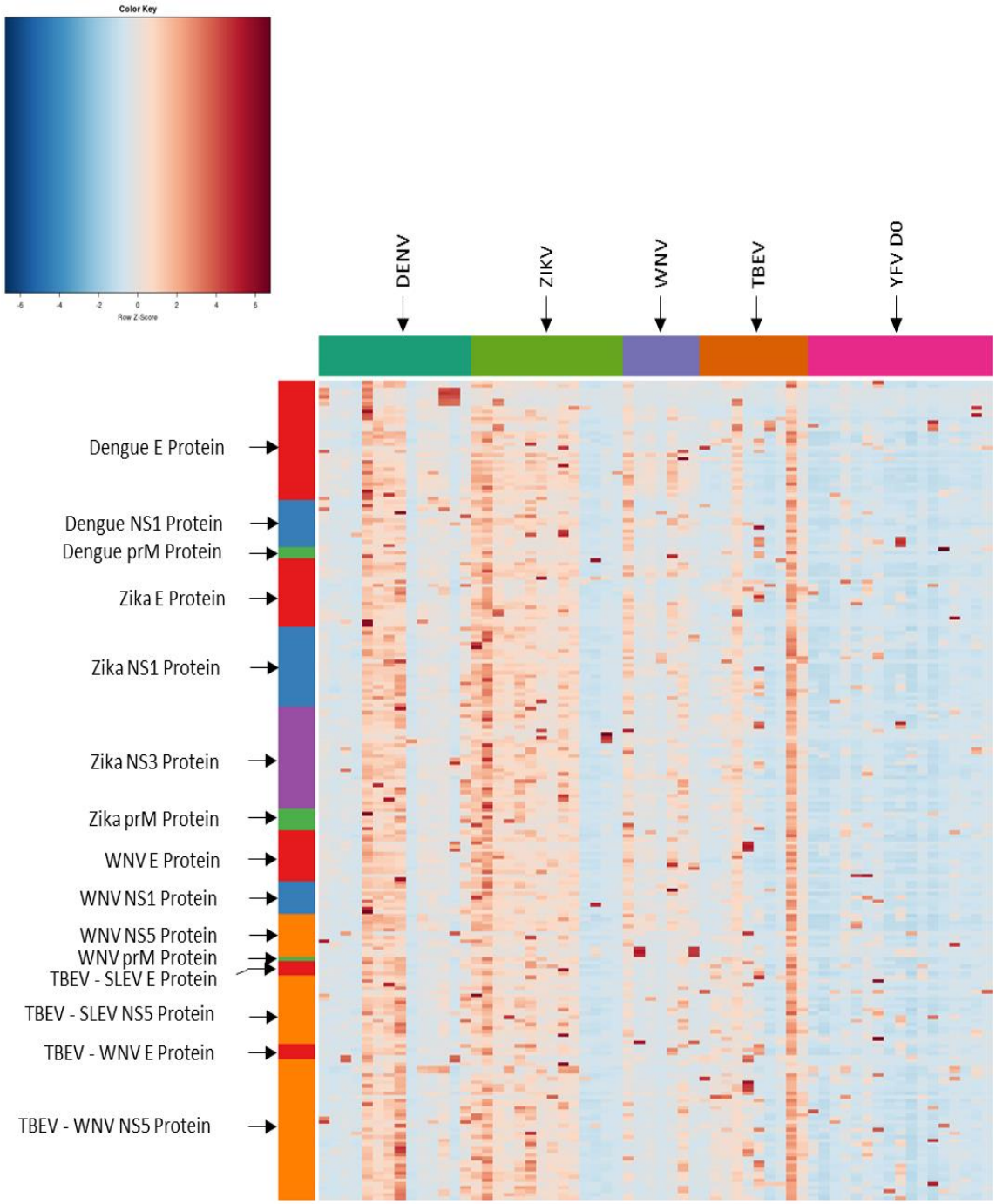


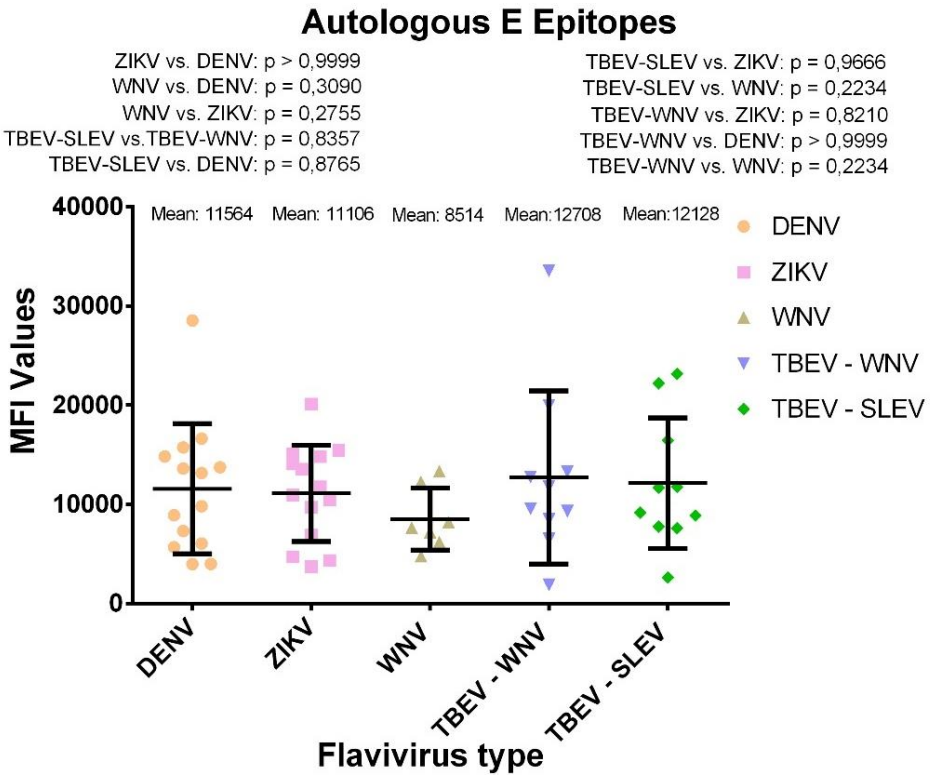
Figure 16 Advanced heat map of all flaviviruses. The different flavivirus-infected study subjects and YFV D0 controls are displayed on the x-axis and plotted against the different epitopes of various proteins on the y-axis. X-axis: different flavivirus study subject samples; y-axis: peptides along the previously selected potential flavivirus-type-specific sequences (N-terminus: bottom) within different flavivirus proteins (sequences not shown in detail); z-score color key displayed on the top left.

Figure 16 shows complete absence of immunodominant regions strongly recognized by all flavivirus-infected study subjects (within one flavivirus & across different flaviviruses). No flavivirus-specific IgG recognition pattern is detectable.

The control group (YFV-D0) in comparison shows weak IgG recognition, but nevertheless, unexplainable high MFI reactivities are seen in some of the selected epitopes.

3.5 Statistics & graphing

The p-values calculated by the GraphPad Prism software (163) according to the nonparametric Mann-Whitney U test (Wilcoxon rank sum test) are shown in the graphs [Figure 17; Figure 18 and Figures S 13- S 16] and revealed mostly non-significant p-values for differentiation between all different sera from flavivirus-infected study subjects.



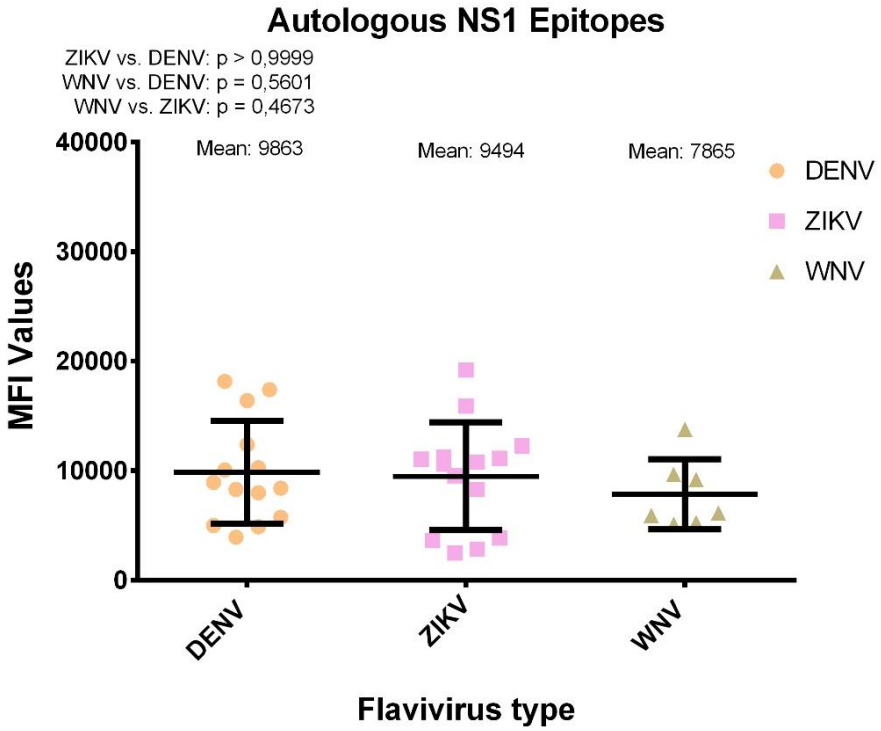
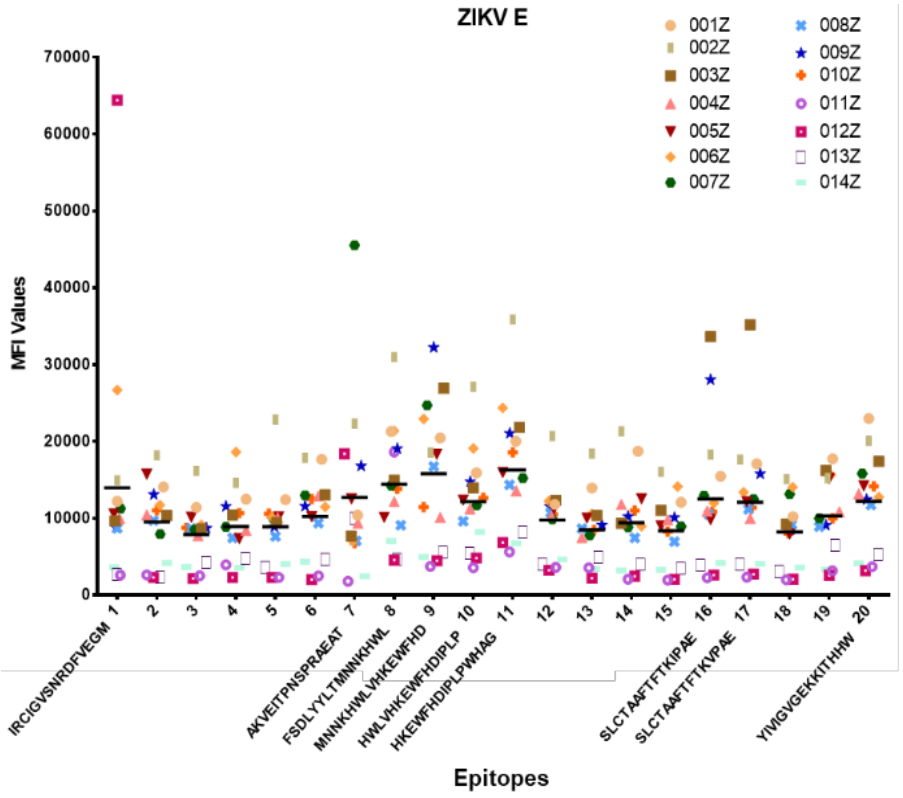


Figure 17 Overview of MFI values of all different sera of flavivirus-infected study subjects against autologous epitopes within E protein (upper graph) and NS1 protein (lower graph). Each study subject is represented by one dot comprising the mean of IgG reactivity (MFI values) against all selected autologous epitopes within E protein (upper graph) and NS1 protein (lower graph). Due to the fact, that TBEV epitopes are not displayed on the array, the IgG reactivity of TBEV-infected study subjects against the selected epitopes within E protein for WNV (TBEV-WNV) and SLEV (TBEV-SLEV) are exemplary shown. X-axis: Flavivirus type; y-axis: MFI values; overall mean of IgG reactivity and non-significant p values displayed above the graph.

The overall mean of IgG reactivity of all sera against previously selected autologous epitopes for each flavivirus were computed for further analyses [Figure 17]. The mean overall IgG reactivity of DENV and ZIKV sera against selected autologous epitopes are very similar, whereas the mean IgG response of WNV sera were lower on average. In general, TBEV-infected study subjects showed higher mean IgG reactivity against selected epitopes within the E protein of WNV than SLEV. Nevertheless, even in the selected epitopes, outliers of MFI values are seen in every flavivirus group, showing a high variability even within study subjects infected against the same flavivirus. To narrow down the selection of epitopes even further and to have a closer look into single epitopes being potentially flavivirus-type-specific, we plotted all MFI values of each study subject of previously selected epitopes shown exemplarily for ZIKV E protein below [Figure 18], where differences between ZIKV and WNV as well as YFV-D0 are considered significant according to p values within previously selected epitopes of ZIKV NS1 ($p < 0.05$) as well as in ZIKV NS1 [Figure S 13].

A further selection was made by calculation of the mean of MFI values of all study subject serum samples for each single epitope. The mean is marked as a black bar for every single epitope. All epitopes where the mean is higher than overall study subject sample mean calculated in Figure 18 were displayed on the graph in form of bar charts representing our final selection of potentially flavivirus-type-specific epitopes. The graphs on flavivirus specificity of further selected epitopes for E, NS1 and NS5 proteins of DENV, ZIKV, WNV and TBEV respectively, can be found in the supplementary material [Figures S 13- S 16]. Data on prM protein are not shown because of inconclusive results. Due to the non-ZIKV specific reactivity of IgG targeting epitopes on NS3 protein seen in advanced heat maps, the graphical analysis was not followed up.



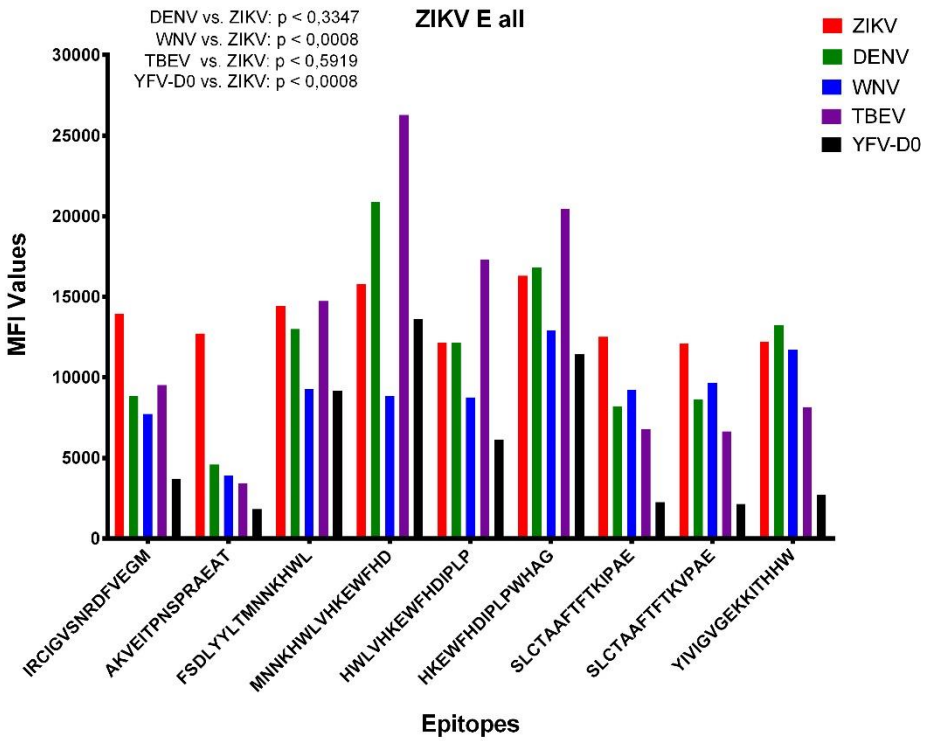


Figure 18 Overview of further selection of epitopes and epitope specificity for ZIKV E protein. The selection is based on calculation of mean for each epitope higher than overall study subject mean [Figure 17]. The selected epitopes in the upper graph were then plotted against mean IgG reactivity of other flavivirus study subjects and control group (YFV-D0) to determine flavivirus-specificity (lower graph). X-axis: different epitopes of protein; y-axis: MFI values; non-significant p values except for YFV-D0 displayed above the right graph.

The interpretation of graphs revealed no flavivirus-type-specific IgG peptide recognition signature between different flaviviruses. The selection of epitopes has mainly been tampered with single MFI value outliers, although p-values show significant difference in WNV and YFV-D0 group compared to ZIKV group. Higher MFI values for single epitopes compared to other epitopes of the same virus and protein does not ultimately lead to higher flavivirus epitope specificity when compared to reactivity of other flavivirus-infected study subjects against epitopes of ZIKV E protein as well as ZIKV NS1 protein [Figure S 13]. Due to the high clinical relevance of distinguishing ZIKV from DENV infection and the high level of cross-reactivity between these observed in most of serological assays (9,12,13,25,48,61,82,85,87,88,115), we selected the most reactive DENV NS1 epitopes in ZIKV study subjects and vice versa by plotting MFI values of frequency of responders (FOR) of >50% against heterologous epitopes in DENV- and ZIKV-infected individuals in a separate graph. Afterwards, we added values of samples reacting against autologous epitopes to show the level of specificity, which has proven to be low [Figure 19].

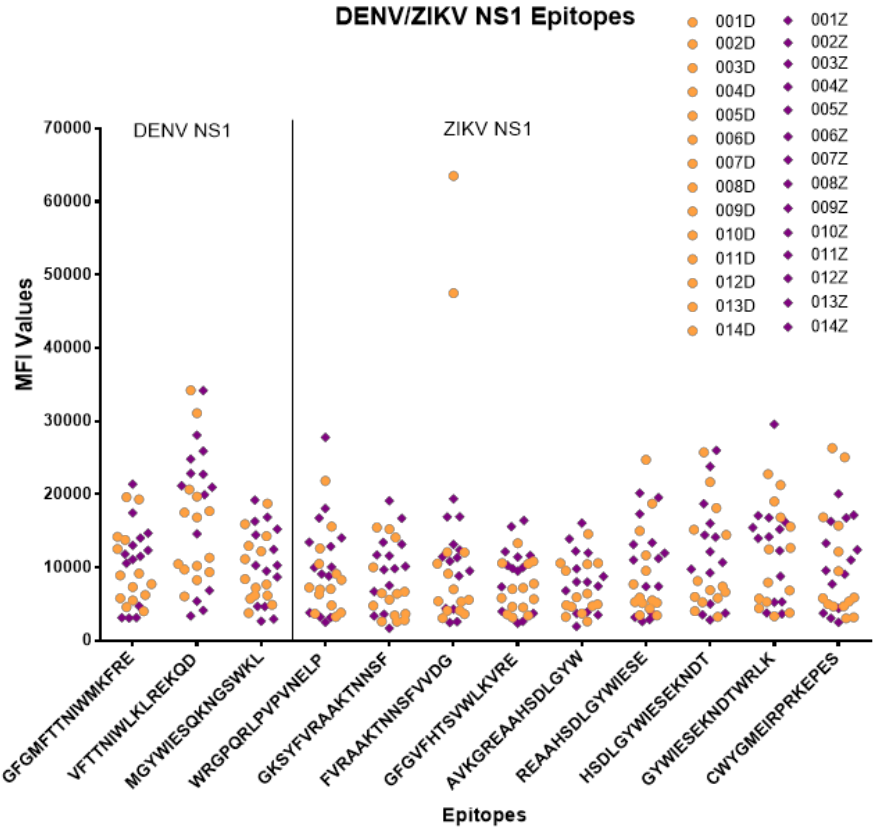


Figure 19 Overview of epitope specificity for DENV epitopes (left side of graph) and ZIKV epitopes (right side of graph) within NS1 protein. High degree of cross-recognition of ZIKV-infected study subjects reacting to previously selected DENV NS1 epitopes and vice versa. X-axis: different epitopes of DENV NS1 (left) and ZIKV NS1 (right) protein; y-axis: MFI values.

4 Discussion

The peptide array method was used in this thesis as a serodiagnostic tool, and our data showed complete absence of flavivirus-type-specific IgG peptide recognition signatures within different flavivirus-types and across flavivirus-infected and YFV-vaccinated study subjects.

The statistical analysis by Mann-Whitney U test revealed non-significant p-values for differentiation between all different sera from flavivirus-infected study subjects, particularly collating DENV and ZIKV sera. The interpretation of p-values in the Mann-Whitney U test is further complicated by different sample sizes of different flaviviruses tested in this thesis. The most probable explanation is a high level of flavivirus Ab cross-reactivity targeting linear epitopes of our peptide array which has been observed in various serological assays among all different flaviviruses.

A disadvantage of the peptide array technology is that it is only able to detect linear epitopes. Indeed, it was suggested by Regenmortel that an intact protein consist of approximately 90-95% discontinuous and only 10% linear epitopes targeted by b cells (164) leading to an almost unrestricted diversity in the Ab repertoire after viral contact (69).

In the study of Viedma et al. (140) peptide array data predicted high-scoring immunodominant epitopes for diagnostics displaying epitopes of DENV I-III, ZIKV, CHIK and WNV. Although five epitopes in DENV I-III, five epitopes in ZIKV and one epitope in WNV identified were also displayed on our array, none of them were included in our final analysis using GraphPad Prism due to lack of IgG reactivity in our serum samples. Whether it is due to their prediction algorithm, or our data analysis remains unclear.

One explanation for the insufficient detection of a flavivirus-specific IgG response could be the age and quality of our peptide arrays, which were printed by JPT Technologies in June 2018. The tests were performed from November 2019 to January 2020. Indeed, when analyzing the signal intensities of our aliquoted negative control sera each month over three months, the signal intensity and background signals of the last serum (Q3) were decreased [Figure S 5a].

A high inter-assay variance can be seen when looking at the different IgG epitope recognition patterns (or rather MFI values) in the YFV E protein. This can be interpreted as batch effects due to background variation which tampered the detection of significant MFI values seen especially in heat maps of study subjects with ZIKV-infection [Figure S 6]. As a reason for this phenomenon, it can be stated that we purposely decided not to carry out background subtraction in our samples as sera from study subjects prior flavivirus infection were not available and a high inter study subject variability of IgG responses before YFV vaccination was observed [Figure 13; Figure S 7]. All in all it is unclear whether pattern relates to infection or rather vaccination or to unspecific background signals. However, low levels of IgG reactivities in negative control sera are also seen elsewhere in peptide arrays (100).

Comparison of ELISA and peptide array results

While peptide arrays and dot blot assays only detect linear epitopes, several epitope mapping techniques including ELISA and Western Blot (WB) assays are not always sufficiently able to differentiate between conformational and linear epitopes. This impedes validation and comparison of our peptide array results with other serodiagnostic approaches further complicated by variation in assay type, antigens, data analysis as well as usage of sera from flavivirus-infected and -vaccinated study subjects.

The design of serological assays to detect recombinant instead of native proteins has been implemented not only in ELISA formats (26,34–37) but also in peptide array in recent years (3). Interestingly, in contrast to our results, a custom-made multiplex serological protein array by Cleton et al. with recombinant NS1 proteins of various flaviviruses, IgG reactivity profiles of DENV, JEV, Usutu virus and WNV could be clearly distinguished in form of heat maps and were mainly directed against their homologous antigens. Additionally, low titers in negative controls and in TBEV and JEV vaccinees were found (62).

While strong ZIKV NS1 protein binding in other serological assays was observed (12,38,61,70), Ab binding in our peptide arrays as well as in Heffron et al. (152) was scattered through epitopes across study subject samples, which leads to the assumption that these NS1 epitopes might be missed in linear epitope assays.

However, strongly reacting ZIKV-specific linear epitopes within NS1 protein with high sensitivities and specificities for sera of ZIKV-infected study subjects compared to DENV and CHIKV have indeed been identified in an indirect ELISA (161). In addition, strong detection of ZIKV reactive IgG Abs against epitopes within ZIKV NS2B protein was seen in high-density peptide arrays in human samples (87,165), while high IgG reactivity was seen for E protein and also NS3 protein in a peptide array using sera of macaques (152).

Another study applied the peptide array assay method to detect seven binding sites of ZIKV-specific polyclonal Abs in the ZIKV E protein, whereas the sequence within one of these sites matched one of our epitopes further analyzed in Figure 18 except for the last amino acid (YIVIGVGEKKITHH(W)). This site was further characterized and identified as a non-/weak-neutralizing epitope, which is partially surface exposed, lays in EDIII region of ZIKV E protein and has a high level of overlap with previously described ZIKV neutralization epitopes (154). Nonetheless, it appeared not to be ZIKV-specific with high MFI values in DENV-infected study subjects in our analysis [Figure 19].

Hansen et al. (153) discovered nine ZIKV-exclusive 15-mer epitopes which were detected by IgG and display ZIKV-specific Ab target regions in E, NS1, NS3, NS4 and NS5 proteins in a custom-made panflavi peptide array. Seven of them were not displayed on the peptide array used in this doctoral thesis, whereas two epitopes (located in NS3 and NS5) were not included in our further analysis due to lack of ZIKV-specific epitope recognition according to our analysis criteria. Another epitope recognized by IgM and IgG within ZIKV E protein (sequence no. 592-SLCTAAFTFTKVPAE) in the study of Hansen et al. was considered cross-reactive (153). In our study, this epitope has shown a high IgG reactivity across all sera of ZIKV-infected study subjects and was suggested to be ZIKV-specific, however IgG reactivity was also seen in other flavivirus-infections [Figure 18].

In contrast, the custom-made array of Bergamaschi et al. identified one epitope spanning 12 amino acids in the domain I of the E protein (sequence DRGWGNGCGLFG) which was able to distinguish Dengue infection from control sera but was also highly cross-reactive to following tests with ZIKV sera (71).

Interestingly, this DENV sequence is included in two epitopes displayed in our array, whereas one (sequence VDRGWGNGCGLFGKG) located in the DENV E protein has proven to be DENV specific in our analysis [Figure S 14] although sequence is also found in SLEV, WNV, ZIKV. The whole studied DENV epitope of Bergamaschi et al. is identical in WNV, JEV and YFV (71), therefore low DENV-specificity is comprehensible. Discrepancies in epitope specificities between computational predictions and serological assays were also observed for peptide-based ELISA (78) and custom-made panflavi peptide array (153).

While IgG responses targeting the prM protein were measured after DENV infection (47,101,102,104), Kam et al. showed the specific homologous IgG recognition of one prM epitope in DENV, as well as one epitope in ZIKV-infection from convalescent DENV and ZIKV patients in a peptide-based ELISA (94). Besides, Abs against prM were able to differentiate Dengue infection from WNV, JEV in Western Blot assays (102,103), which couldn't be confirmed by our results for prM in different flavivirus-infections. However, data showed high degree of cross-reactive Abs elicited against prM, also involved in ADE (36,45).

The study of Amrun et al. identified thirteen potential linear B-cell epitopes in different flaviviruses in form of a peptide-based ELISA (78). Of these, two epitopes were part of epitopes included in our final analysis (sequence GKSYFVRAAKTNNSF within ZIKV NS1 and sequence YRPGYHTQTAGPWHL within DENV NS1). Interestingly, the first epitope located in ZIKV NS1 showed similar binding capacity between DENV and ZIKV steadily over time (78), which is concordant to our results that this surface-exposed epitope showed reduced specificity for ZIKV NS1 protein [Figure S 13]. However, it was declared ZIKV-specific (159,161). The other epitope located in DENV NS1 was identified as potentially DENV-specific with a high level of corresponding sequence similarity and difference in binding capacity compared to ZIKV, which could not be seen in our graph [Figure S 14].

Preexisting flavivirus immunity

The impact of preexisting flavivirus immunity on Ab specificity resulting in higher concentrations of broadly flavivirus cross-reactive IgG Abs was shown in individuals with prior TBEV and/or YFV vaccination compared to flavivirus-naïve study subjects after ZIKV infection (166). Differences in IgG reactivity between a group of endemic DENV2-infected individuals and a pool of DENV-infected travelers were also observed in a peptide microarray by Falconi et al. High IgG reactivities were aimed at regions within C, E and NS3 protein in the endemic group, whereas IgG recognition was low or missing in the traveler group regarding the same regions (100). Therefore, the observed high variance of study subject epitope reactivities within members of the same serocomplex or even virus type in our study can potentially be explained by the fact that most of the sera from study subjects used in this study miss a complete vaccination record and a detailed travel history.

However, for ZIKV and DENV study subjects as well as for YFV vaccination study participants, all samples (except from one Zika-infected study subject from Peru) used in this thesis were returning travelers born in Europe. Travel history of the YFV vaccination cohort included Europe, southern Africa and Oceania, but deliberately did not include YFV endemic regions. However, a high, reactivity was detected in one YFV serum 005_Y at both timepoints D0 and D28. This remains inexplicable as the study participant had a negative travel record for >2months outside Europe and no previous TBEV oder JEV vaccination.

The low general IgG response of WNV sera seen in Figure 17 can be partly explained by limited information on study subjects especially concerning method of virus infection identification but also timing of sampling.

Early vs. late convalescent

Concerning ZIKV and DENV sera used in our study, information on time interval between symptom onset and sample collection was gathered [Figure S 1; Figure S 2].

For ZIKV-infection, serum samples were collected after seroconversion from an interval ≥ 8 days with rising immune fluorescence antibody test (IFAT) IgG reactivities for ZIKV and other flaviviruses until a 42-day interval where high ZIKV IgG responses are still measurable. While most of the DENV study subject sera were collected in the convalescent or postconvalescent phase, six samples were collected three to five days after symptom onset in acute phase of the disease where - concordant to Ab kinetics - IgG Abs haven't been developed yet and therefore aren't measurable (65). In the acute phase of DENV disease, a higher variety in IgM Ab responses throughout the whole proteome was found compared to IgG responses lasting until minimum of 6-month post illness (107). Surprisingly in the same peptide array study, the IgG response directed against YFV and ZIKV peptides in convalescent sera from DENV-infected travelers was broader than compared to IgM responses. The overall number of targeted IgG Abs in DENV2 infected individuals raised from acute to early-convalescent phase of the disease and declined thereafter in late-convalescent phase (100). This is partly reminiscent of our data, where weak IgG reactivities against DENV, TBEV, JEV, WNV, YFV and ZIKV were already measurable after an interval of 4 days in IFAT and heat maps.

All in all, a high variance in IgG responses against DENV and ZIKV in comparison to other flaviviruses in IFAT as well as in our peptide array is only partly explainable by intervals of sample collection [Figure 12; Figure S 6]. No cluster of IgG responses in peptide array heat maps can be seen when differentiating between early convalescent sera (≤ 14 days interval between onset of symptoms and sample collection) and late convalescent sera (> 14 days interval).

In literature however, the peptide array performance of early vs. late convalescent phase sera differed as flavivirus-specific IgG responses are more likely to be detected in early convalescent phase sera (2-3 weeks after infection) than 6 months post infection in ZIKV (87).

For DENV, Hertz et al. identified immunodominant peptides in epitopes of NS1 proteins in naturally infected humans (150) in peptide array concordant to similar results in mice (167). Stronger Ab responses against DENV NS1 protein were detected in convalescent phase compared to acute phase and at 12-mo timepoint (150).

Interestingly, some Ab responses against strongly recognized regions in convalescent phase waned at 12-month timepoint, which has been seen in ZIKV-infected macaques as well (152). The strongest Ab response was measured in early convalescence (96%), whereas it declined to 44% at 6-months post infection. These findings in peptide arrays stand in line to results of the NS1-BOB ELISA study of Balmaseda et al. for ZIKV, where sensitivity was increased at >20 days post-onset of symptoms compared to >10 days post symptom onset (61).

Diagnostics of flaviviruses is also complicated because of varying persistence of detectable Abs in different sample types (serum, urine etc.) (168). This can potentially lead to reduced viremia and slower Ab kinetics especially in anti-NS1 Abs in ZIKV infection compared to e.g. CHIKV and DENV infection (3,14). This study however, observed PCR-positivity in whole blood after 16 days interval between symptom onset and sample collection (sample 011_Z); PCR positivity in whole blood and high Ab titers after interval of 9 days (sample 007_Z) as well as a general high IgG response in most of ZIKV-infected study subjects [Figure S 1]; although sera of study subjects with DENV infection were collected at an earlier timepoint after symptom onset than from ZIKV-infected individuals.

ZIKV vs. DENV

Arboviral serodiagnostic tools have been developed mainly for ZIKV and DENV and the correct serodiagnosis still poses great challenges. In the PCR and IFAT results of our DENV and ZIKV sera used subsequently in peptide arrays, almost all samples had a high level of cross-reactivity in IgG titers across DENV, ZIKV, TBEV, JEV, WNV and YFV.

One reason could be that ZIKV has the highest amino acid identity (55,6%) with DENV compared to other flaviviruses (169) and one paper proposed to include the ZIKV in the DENV serocomplex as a fifth member due to high cross-reactivity (9).

Additionally, study subjects travel histories to different countries as well as the geographical cocirculation of different arboviruses (e.g. DENV and ZIKV) can lead to an overlap in decreasing and declining Ab responses to independent infections with the tendency to cross-react (3).

The comparison of IgG titers of non-endemic (travelers) and individuals from multiple DENV endemic countries showed significant results ($p<0.01$) (62). The occurrence of cross-reactivity in secondary DENV or ZIKV infection has already been observed in other serological assays (11,25,47,52,61,85,88). Besides, a high degree of cross-reactivity occurring especially using whole viruses (3,87) as well as in prior ZIKV-infected macaques challenged with DENV infection (152), an additional time-dependent dynamic component of cross-reactivity was detectable (49,52).

These results resonate with our findings that especially ZIKV and DENV infections are difficult to distinguish] for responses against NS1 protein [Figure 19]. A recent study included inter alia 10 DENV immunodominant peptides within prM, E, NS1, NS2b, NS3, NS4b, and NS5 proteins recognized by IgG previously identified via peptide microarray (100) in a bead-based multiplex peptide immunoassay. Best serodiagnostic capacity (sensitivity of 73%) was reached with a peptide within E protein when testing DENV sera from acute patients (<8 days since symptom onset), while sensitivity of 86% was achieved with sera from convalescent DENV patients (>10 days since symptom onset) using a peptide located in NS1 protein. Overall, the combination of peptide biomarkers was proven most successful for discrimination of flavivirus infection and vaccination (170).

Cross-reactivity between DENV serotypes

We purposely used sera from study subjects infected with three different DENV serotypes (DENVI, II, IV) on a peptide array comprising peptides of all four DENV serotypes to compare Ab kinetics and cross-reactivity between these. Besides the missing IgG response pattern against autologous DENV across all samples, we couldn't dissect the immune response into the different serotypes I, II and IV, which has already been apparent comparing IgG DENV IFAT results [Figure 12; Figure S 2].

This corresponds to data acquired by peptide array in another study, where high IgG responses targeting DENV2 peptides specifically were missing in DENV2-infected individuals (100). A high degree of cross-reactivity between heterologous serotypes has been observed in DENV-infected NHPs (3).

In Cleton et al. (62), one proportion of DENV-infected individuals developed high IgG titers against multiple serotypes, whereas the majority showed highest titers against homologous serotype. All in all, epitope recognition patterns varied across humans infected with different DENV serotypes (3,51,62,150,152). This may be due to different levels of structural conservation between serotypes resulting in slight amino acid differences and different serotype-specific Ab binding affinities.

Yellow fever virus

It is known, that after YFV vaccination a low level of viremia is measurable mimicking a YFV infection. Accordingly specific Ab titers are raised which seemed to be lower than in natural infection (120). After vaccination, neutralizing Abs appear after ten to fourteen days and 99 – 100% were seropositive on day 28 after vaccination in PRNT (171), therefore we anticipated an increase of IgG responses at timepoint D28 in our peptide arrays accordingly.

In contrast to our expectations, D0 sera of a subset of our 18 YFV-vaccinated study subjects showed unexplainable IgG responses against antigens of YFV, which are also seen 28 days after vaccination (D28). In general, a low degree of Abs raised against the live-attenuated vaccine compared to our flavivirus sera was observed. Additionally, the IgG response in the YFV vaccination cohort has shown to be independent from YFV vaccination (D0 vs. D28), TBEV-vaccination (non TBEV-vaccinated vs. TBEV-vaccinated) and age [Figure 13]. These results are in accordance with findings from the study of Zumarán, which was not successful in measuring Abs against recombinantly expressed GST-X-tag fusion proteins in the same yellow fever vaccination cohort samples used in this thesis (172). High individual variability of Ab specificities to YFV vaccination for E and prM protein as well as their influence on virus neutralization are shown in different immunoassays (18).

Nevertheless, YFV-specific Ab responses in postvaccination sera from the same yellow fever vaccination cohort samples used in this study have recently been detected in a parallel study via ELISA. Whether these Ab responses binding recombinant E protein as well as the whole virus particle of YFV-17D target linear epitopes remain unknown.
Interestingly, in YFV-17D-naïve sera from individuals with prior TBEV vaccination cross-reactive panflavi ab responses targeting especially the YFV-17D fusion-loop epitope of E protein was detectable and boostered following YFV-17D vaccination. This stands in contrast to the absence of cross-reactive panflavi ab responses in sera of individuals after YFV-17D vaccination and no history of TBEV vaccination (data not yet published). Cross-reactive DENV IgG Ab titers especially in patients with previous TBEV immunization or infection were measured in ELISA by Allwinn et. al (115). These results are further supported by Houghton et al. showing high IgM and IgG Ab cross-reactivity in Dengue ELISA in postvaccination YFV samples (17). The development of cross-reactive IgG in YFV vaccinees prior exposure to other flavivirus antigens (68) is concordant with results described elsewhere (22). A recent study showed interference of preexisting YFV vaccine-derived immune sera with the neutralizing Ab response to TBEV vaccination as well as boosting of cross-reactive, non-neutralizing Abs (130). Kayser et al. showed that predominantly anti-TBEV IgG Abs appeared earlier and in higher titers in the group of prevaccinated YFV patients after administration of TBEV in HI assay than in the control group without previous vaccination (23). This phenomenon wasn’t observed in our study with YFV-vaccinated individuals prior TBEV-vaccination and also not in study of Zumarán (172), where MFI values for YFV E antigens in previously TBEV-vaccinated subjects were partly even lower than in non TBEV-vaccinated. Therefore, no significant cross-reactivity can be concluded.

Concerning Ab responses against YFV antigens in peptide arrays, serum Abs of nonhuman primates (NHPs) vaccinated with YF-17D mainly targeted YFV E protein but also the E protein of ZIKV and TBEV (23), while others observed that YFV NS1 was highly reactive in vaccinated individuals (62).

This stands in contrast to preliminary results from Monath et al. indicating that NS1 Abs do not appear after primary 17D vaccination (120).

One explanation for low MFI values in our array in postvaccination samples could be lack of sensitivity of IgG Abs, as predominantly IgM responses are expected after a few weeks which might interfere with IgG recognition. Low protecting titers of other live attenuated vaccines like measles have been observed and could have tampered our results in our YFV vaccination cohort (108). Besides, IFA and PRNT results demonstrate a stronger immune response in males compared to females after YFV vaccination (68), an observation made in measles-mumps-rubella vaccine as well (173). However, a gender-specific IgG immune response could not be established in our data [Figure 13], but only 6 out of 18 YFV-vaccinated individuals we tested were male [Figure S 3].

The analysis of age-related differences in the b-cell receptor repertoire structure of plasma B cells in young (19–26 years old) versus middle-age (45–58 years old) individuals suggest that younger individuals react with a more diverse Ab response than elder individuals to YFV vaccine (174). This observation cannot be confirmed in our peptide array for detection of IgG Ab responses.

Concerning the analysis of TBEV sera, the study of Kuivanen et al. identified epitopes in E and NS5 protein via SPOT peptide array which reacted in most TBEV-infected patients. Two epitopes in E protein (located in domain I and domain II and were part or form conformational epitopes) raised Ab signals only in TBEV-infected patients and not in vaccinated ones as well as in WNV and DENV sera. Additionally, a few epitopes located mainly in the E protein which reacted in sera of TBEV-vaccinated individuals and not in TBEV-infected or TBEV-seronegative study subjects were found (151). This indicates that the vaccine inactivation process modifies IgG reactivity against these peptides. TBEV antigens were not displayed on our array, which complicates the application of these results on our findings. Nevertheless, concerning antigenicity against epitopes of WNV E protein, we observed weak epitope recognition across all TBEV-immunized study subjects compared to TBEV-infected study subjects without specific IgG signatures [Figure 14].

An NS5-based immunoassay has previously shown distinctive capacities of WNV infection against DENV- and SLEV-infections as well as flavivirus-vaccination (89). Nevertheless, no IgG-specific signature was seen in our analyses for TBEV-infected study subjects reacting against WNV and SLEV NS5 epitopes [Figure S 10] as well as in WNV-infection [Figure S 11].

CHIKV antigens were also mapped on the array, which represent an alphavirus more distant to flaviviruses. It was therefore thought to not elicit any Ab response in flavivirus-infected study subjects. However, similar to our observations (data not shown) and other reports in ELISA (but not in peptide array (87)), cross-reactivity occurred. No Ab responses were detected in previous analyses of ZIKV- and DENV sera used in this thesis via IFAT against CHIKV antigens [Figure S 1;Figure S 2].

In general, dissecting the flavivirus Ab response is complex and IgG cross-reactivity in serological assays widely occurs. This is in line with results obtained by our pan-flavivirus peptide array tested with a panel of sera from flavivirus-infected and YFV-vaccinated study subjects. Nevertheless, peptide arrays have proven previously to be a valuable method for detection of a flavivirus-specific IgG response with higher sensitivity and specificity compared to ELISA (87). Additionally, the use of peptide arrays in human immunodeficiency virus (HIV) infection as well as vaccination has shown promising results (156). The immunogenic V3 region in HIV shows continuous IgG recognition across various HIV vaccine trials [paper accepted, (175)].

Further studies are necessary for identification of both species-specific and pan-arbovirus antigens utilized for the development of a more specific diagnostic test.

This study points out the difficulties in detecting flavivirus-specific immune responses via peptide array method but paves the way for a more detailed in-depth epitope mapping and a further understanding of Ab responses in flavivirus infections.

Outlook

One approach for future diagnostical array design could be the development of a more specific peptide array regarding computationally identified promising flavivirus-specific epitopes which has successfully been applied before (44,70,71,78,169,176). Another idea is the co-representation of two linear peptides on a “clickable” microchip surface (160) as well as the detection of neutralizing Abs via Fluorescence Reduction Neutralisation Test (FluoRNT) against YFV-17D and via flavivirus reporter replicon particles (177,178). Moreover, the number of amino acids forming an epitope can range between 5 to 22 (179), with residues not ultimately closely connected with the paratope in the antibody. Assays to analyze epitopes of different amino acid length could further cluster Ab binding and immune responses.

One of the biggest future challenges are the global growth of human populations with an increase of urbanization, destruction of unspoiled habitats, climate change, further mutation in the viral structure, problems in effective mosquito control as well as increased travel and globalization (180). Therefore, a future emergence of flaviviruses is expected, which reflects the overriding importance of further research and development of (multiplex) serology and specific, high throughput diagnostical assays (41,181). Particular attention must be paid to the most affected regions in the world and many times limited testing capacities e.g., by the invention of rapid diagnostic test especially for ZIKV and DENV diagnostics (182).

Vaccine development is highly dependent on efficient tools for diagnosis and immune monitoring; but also, for instance, on advancement of peptide arrays to study different experimental ZIKV vaccines (183). Apart from the rapid and successful design of a mRNA-based vaccine in the face of the ongoing SARS-CoV-2 pandemic, mRNA vaccines are developed for TBEV, DENV, ZIKV, and Powassan virus as well (184).

All in all, data collected in this study gives new insights on dissecting the flavivirus immune responses to meet these future challenges.

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Without all of you it would not have been possible to conduct this research and to write this doctoral thesis.

Supplementary material

ID	Age (5 Year-Cluster)	Sex	Country of Origin	Interval (in days)	ZIKV-PCR Serum	ZIKV-PCR Whole Blood	DENV inh Serum	DENV inh Whole Blood	CHIKV-PCR Serum	ZIKV IgM	ZIKV IgG	DENV IgM	DENV IgG	YFV IgM	YFV IgG	TBEV IgM	TBEV IgG	WNV IgM	WNV IgG	JEV IgM	JEV IgG	CHIKV IgM	CHIKV IgG
001_Z	30-34	f	P	10	nd	nd	nd	nd	nd	+	-	+++	-	+++	-	++	-	-	+++	-	+++	-	-
002_Z	40-44	m	G	14	nd	nd	nd	nd	nd	++	++	+++	-	+++	-	+++	-	-	+++	-	+++	-	-
003_Z	30-34	m	G	30	nd	pos	nd	neg	nd	+	+++	+++	-	+++	-	+++	-	-	+++	-	+++	-	-
004_Z	50-54	f	G	12	neg	pos	neg	neg	nd	++	+++	+++	++	+++	+	+++	+	-	+++	-	+++	-	-
005_Z	15-19	f	G	10	neg	pos	neg	neg	nd	+	+++	+++	-	+++	-	+++	-	-	+++	-	+++	-	-
006_Z	30-34	m	G	42	neg	neg	neg	neg	nd	+	+++	+++	-	+++	-	+++	-	-	+++	-	+++	-	-
007_Z	25-29	f	G	9	nd	pos	nd	neg	nd	++	++++	++++	-	++++	-	+++	-	-	++++	-	++++	-	-
008_Z	20-24	f	G	15	nd	pos	nd	neg	nd	++	+++	-	-	-	-	-	-	-	-	-	-	-	-
009_Z	25-29	m	A	10	nd	pos	nd	nd	nd	++	++++	+++	-	-	-	-	-	-	+++	-	+++	-	-
010_Z	30-34	f	G	32	nd	neg	nd	neg	nd	+	+++	+++	-	+++	-	+++	-	-	+++	-	+++	-	-
011_Z	30-34	m	G	16	nd	pos	nd	neg	nd	++	+++	+++	-	+++	unsp	+++	-	-	+++	-	+++	-	-
012_Z	50-54	f	G	21	nd	neg	nd	neg	nd	++	+++	+++	-	+++	unsp	+++	+	unsp	+++	unsp	+++	unsp	-
013_Z	45-49	f	S	8	neg	pos	neg	neg	neg	++	++	-	-	-	-	+	+	+	+	-	-	-	-
014_Z	60-64	m	G	13	nd	pos	nd	neg	nd	++	+++	+++	-	+++	-	+++	-	+++	+++	-	+++	-	-

Figure S 1 Detailed table of sera from ZIKV-infected study subjects. Previous serological diagnostics were carried out with PCR (dark grey) and IFAT (red). m: male; f: female; P: Peru; A: Austria; S: Switzerland; G: Germany; Interval: time point of sample collection after symptom onset; inh: in-house PCR; nd: not done; neg: negative; pos: positive. -: <1:10; +: Range between 1:10 to 1:40 to 1:80; ++: Range between 1:160 to 1:320 to 1:640; +++: Range between 1:1280 to 1:2560 to 1:5120; ++++: Range between 1:10240 to 1:20480; unsp: unspecific, not reliably measurable.

ID	Age (5 Year-Cluster)	Sex	Country of Origin	Interval (in days)	ZIKV-PCR Serum	ZIKV-PCR Whole Blood	DENV inh Serum	DENV inh Whole Blood	CHIKV-PCR Serum	ZIKV IgM	ZIKV IgG	DENV IgM	DENV IgG	YFV IgM	YFV IgG	TBEV IgM	TBEV IgG	WNV IgM	WNV IgG	JEV IgM	JEV IgG	CHIKV IgM	CHIKV IgG	Type	Diagnostics
001_D	30-34	m	G	24	nd	nd	nd	nd	nd	-	++++	++	-	+++	-	++++	-	++++	-	++++	-	-	-	DENV 1	
002_D	45-49	m	G	3	neg	nd	pos	nd	nd	-	nd	+	++++	nd	-	nd	-	nd	-	nd	-	-	nd	DENV 2	
003_D	60-64	f	G	4	neg	nd	pos	nd	neg	-	+	-	+	-	-	+	-	+	-	-	-	-	-	DENV 2	
004_D	30-34	m	G	5	neg	nd	pos	nd	neg	-	-	-	-	-	+	-	-	-	-	-	-	-	-	DENV 2	NS1 Ag & IgM pos.
005_D	15-19	f	G	28	nd	nd	nd	nd	nd	-	++++	+	-	-	++++	-	-	++++	-	++++	-	-	-	DENV 2	
006_D	35-39	f	G	11	neg	nd	neg	nd	nd	-	++++	+	++++	-	++++	-	-	++++	-	++++	-	-	-	DENV 4	
007_D	20-24	f	G	>15	nd	nd	nd	nd	nd	-	+++	++	+++	-	+++	-	-	+++	-	+++	-	-	-	DENV2	
008_D	30-34	m	G	10	neg	nd	neg	nd	nd	-	-	+	++	-	-	-	-	-	-	-	-	-	-	-	DENV 2	
009_D	35-39	f	G	4	neg	nd	pos	nd	neg	nd	-	+	nd	nd	-	nd	nd	-	nd	nd	nd	nd	nd	DENV 1	
010_D	20-24	m	G	13	nd	nd	nd	nd	nd	-	+++	+	+++	nd	-	+++	nd	+++	nd	+++	-	+++	-		Dengue IgM, IgG pos.
011_D	25-29	m	G	5	neg	nd	pos	nd	neg	-	+++	+	+++	-	-	+++	-	+++	-	+++	-	+++	-	DENV 2	Dengue IgM, IgG pos.
012_D	15-19	m	G	15	neg	nd	nd	nd	nd	-	+++	+	+++	-	+++	+++	-	+++	-	+++	-	+++	-	DENV2	
013_D	26-30	f	G	9	neg	neg	pos	pos	nd	unsp	++++	unsp	++++	unsp	++++	++++	unsp	++++	unsp	++++	unsp	++++	-	DENV 2	
014_D	30-34	m	G	5	neg	neg	pos	pos	neg	-	++++	-	++++	-	++++	++++	-	++++	-	++++	-	++++	-	DENV 4	

S 2 Detailed table of sera from DENV-infected study subjects. Previous serological diagnostics were carried out with PCR (dark grey), IFAT (red) and commercially available ELISA (light grey). m: male; f: female; G: Germany; Interval: time point of sample collection after symptom onset; inh: in-house PCR; nd: not done; neg: negative; pos: positive. -: <1:10; +: Range between 1:10 to 1:40 to 1:80; ++: Range between 1:160 to 1:320 to 1:640; +++: Range between 1:1280 to 1:2560 to 1:5120; ++++: Range between 1:10240 to 1:20480; unsp: unspecific, not reliably measurable.

a

ID	Age (5 Year-Cluster)	Sex	Country of Origin/Ethnicity	Flavivirus Vaccination History	PRNT Titer D0
001_Y	35-39	m	G	-	n/a
002_Y	30-34	m	G	-	n/a
003_Y	35-39	f	G	-	n/a
004_Y	30-34	m	G	-	n/a
005_Y	35-39	f	G	-	n/a
006_Y	15-19	f	G	-	n/a
007_Y	20-24	m	G	-	n/a
008_Y	15-19	f	G	-	n/a
009_Y	15-19	m	G	-	n/a
010_Y	15-19	f	G	-	n/a
011_Y	30-34	m	G	TBEV	1:320
012_Y	25-29	f	G	TBEV	1:640
013_Y	25-29	f	G	TBEV	1:160
014_Y	40-44	f	G	TBEV	1:160
015_Y	30-34	f	G	TBEV	1:40
016_Y	30-34	f	G	TBEV	1:10
017_Y	20-24	f	G	TBEV	1:160
018_Y	20-24	f	G	TBEV	1:160

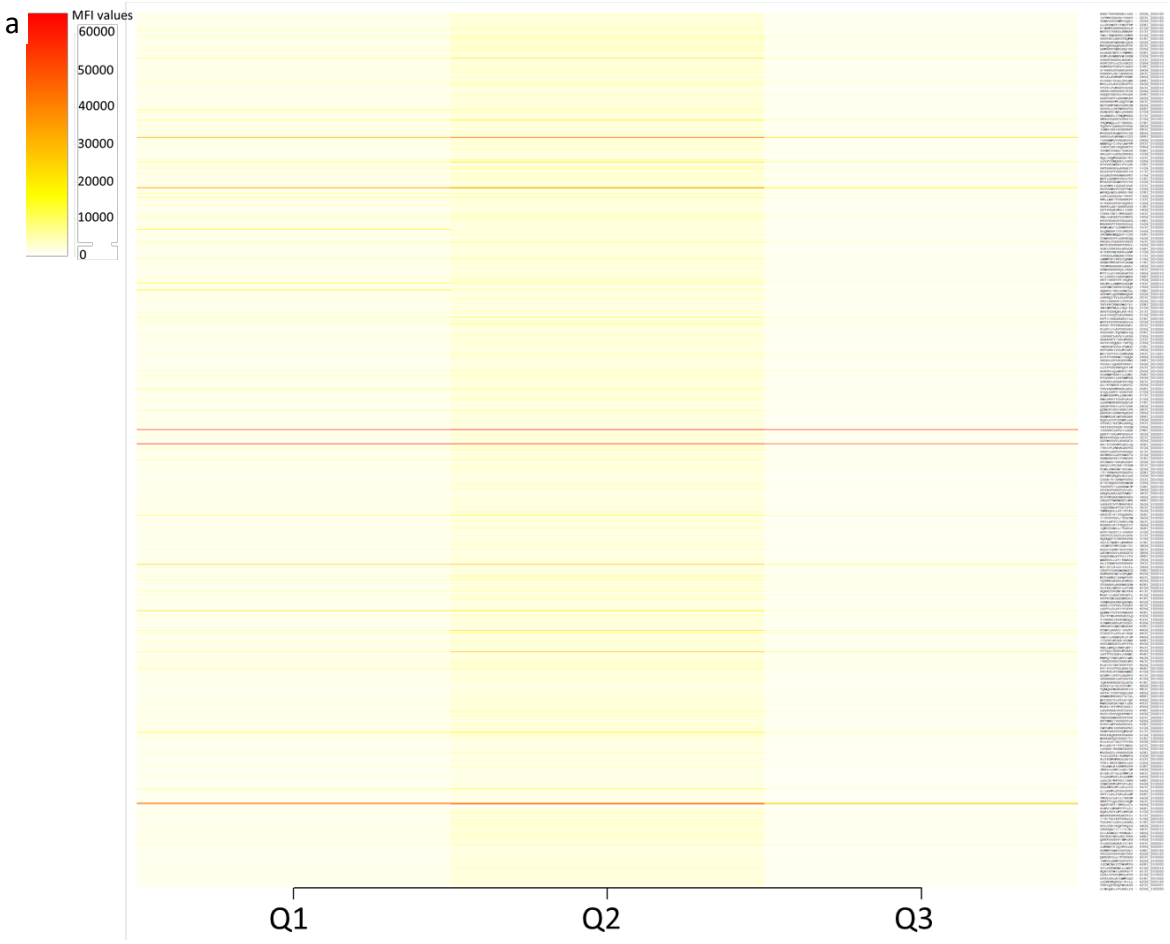
b

Selection of YFV Study Subjects						
		Age (25-44 years)		Age (15-24 years)		Total Number of Samples
TBEV-vaccinated	TBEV PRNT Titer D0	≥1:160	≤1:40	≥1:160	≤1:40	8 = 44,4%
	Number of samples	4	2	2	0	
Non-TBEV-vaccinated	TBEV PRNT Titer D0	n/a		n/a		10 = 55,6%
	Number of samples	5		5		
Total number of samples		11		7		18

Figure S 3 Detailed table of sera from YFV-vaccinated study subjects. a) overview of selected samples b) single study subject information. M: male; f: female; G: Germany.n/a: not available.

ID	Age (5 Year-Cluster)	Sex	Country of Origin/Ethnicity
001_W	35-39	m	Not Hispanic or Latino
002_W	65-69	f	Not Hispanic or Latino
003_W	55-59	f	Not Hispanic or Latino
004_W	60-64	m	Not Hispanic or Latino
005_W	40-44	m	Not Hispanic or Latino
006_W	50-54	f	Not Hispanic or Latino
007_W	40-44	m	Hispanic or Latino

Figure S 4 Detailed table of sera from WNV-infected study subjects. M: male; f: female.



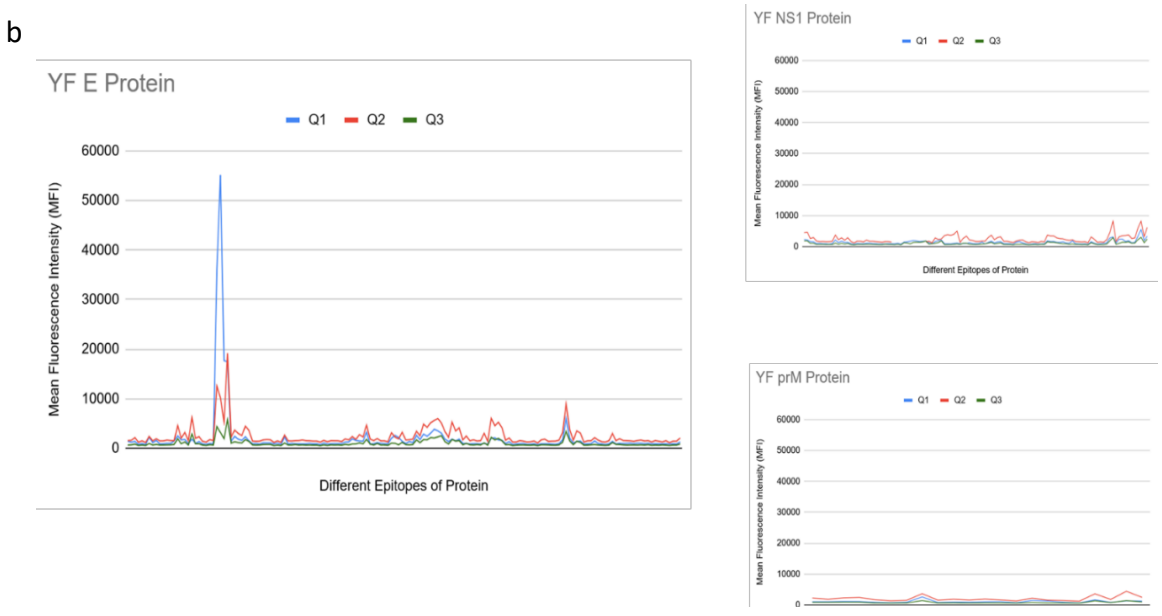
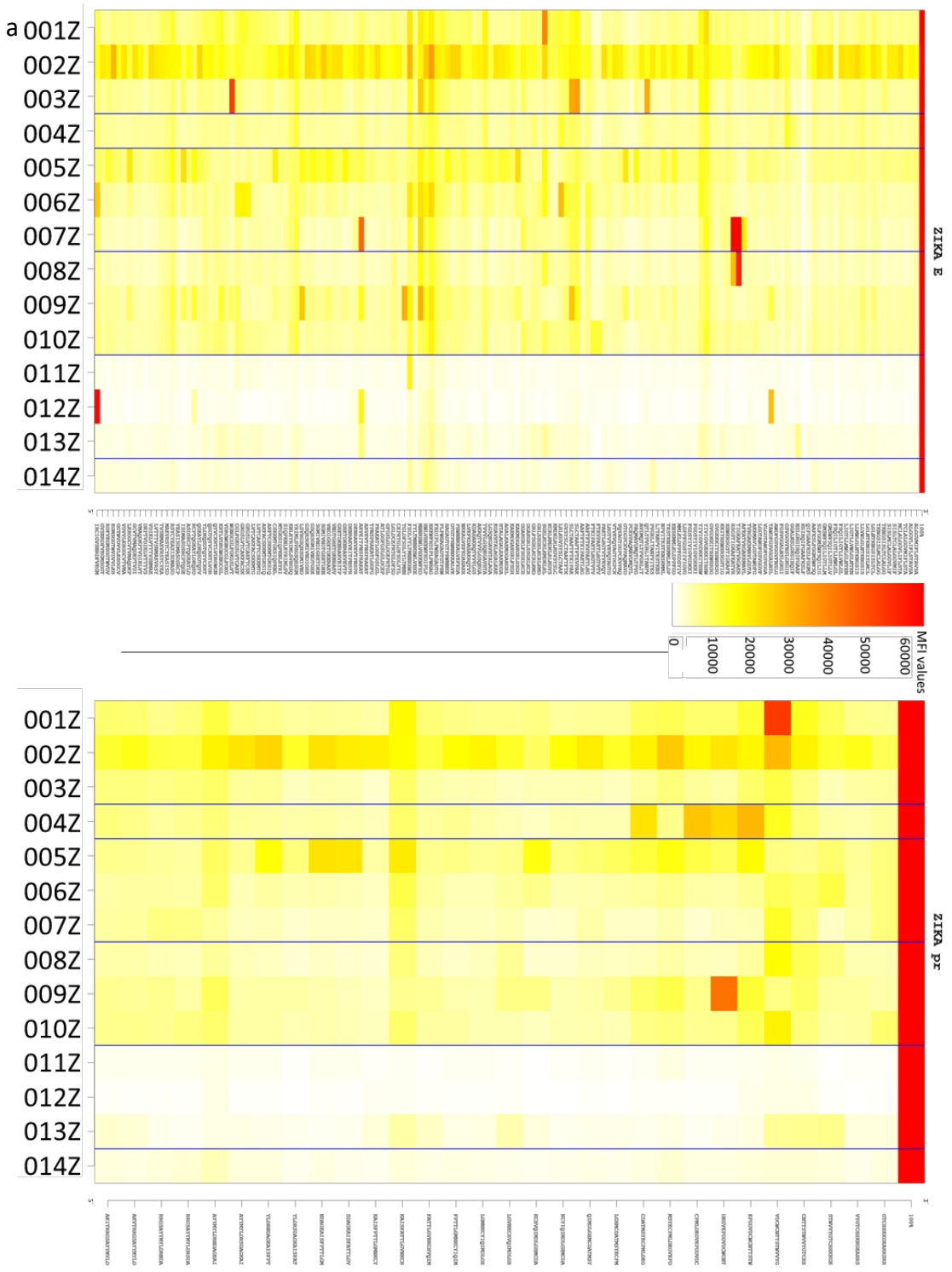


Figure S 5 Aliquots of quality control sera (Q1, Q2, Q3) from one YFV study subject at D28 tested each month during laboratory work alongside other study subject samples. a) heat map of different quality control sera on x-axis plotted against all 6253 peptides on y-axis. Continuous IgG recognition across all 3 quality sera, although the backgrounds of each sample differ. X-axis: quality control sera tested each month during laboratory work; y-axis: peptides along the sequence (N-terminus: bottom); legend to MFI values located on the left. b) detailed analysis of YFV proteins (E, NS1, prM). X-axis: different epitopes of Protein; y-axis: MFI values.


a
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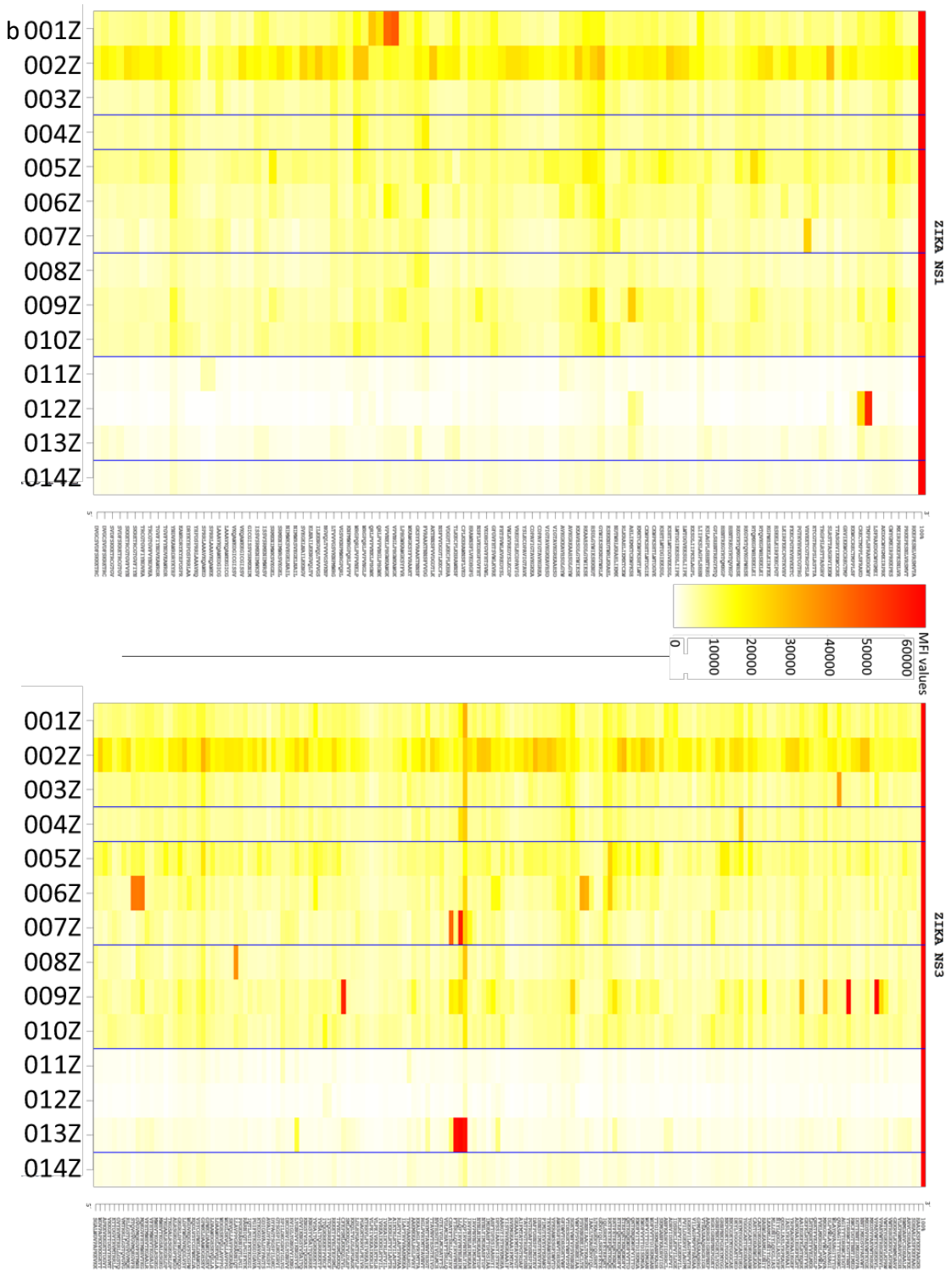

Figure S 6 Color-coded heat map of ZIKV infection samples. Mean fluorescent intensities (MFI) of ZIKV samples on x-axis plotted against all the different peptide sequences mimicking epitopes of the ZIKV E protein (a, upper graph) and ZIKV prM protein (a, lower graph) as well as ZIKV NS1 protein (b, upper graph) and NS3 (b, lower graph). Striking is the strong background in samples 001_Z-010_Z compared to samples 011_Z-014_Z. X-axis: study subject sera; y-axis: peptides along the sequence (N-terminus: bottom); legend to MFI values located in the center.

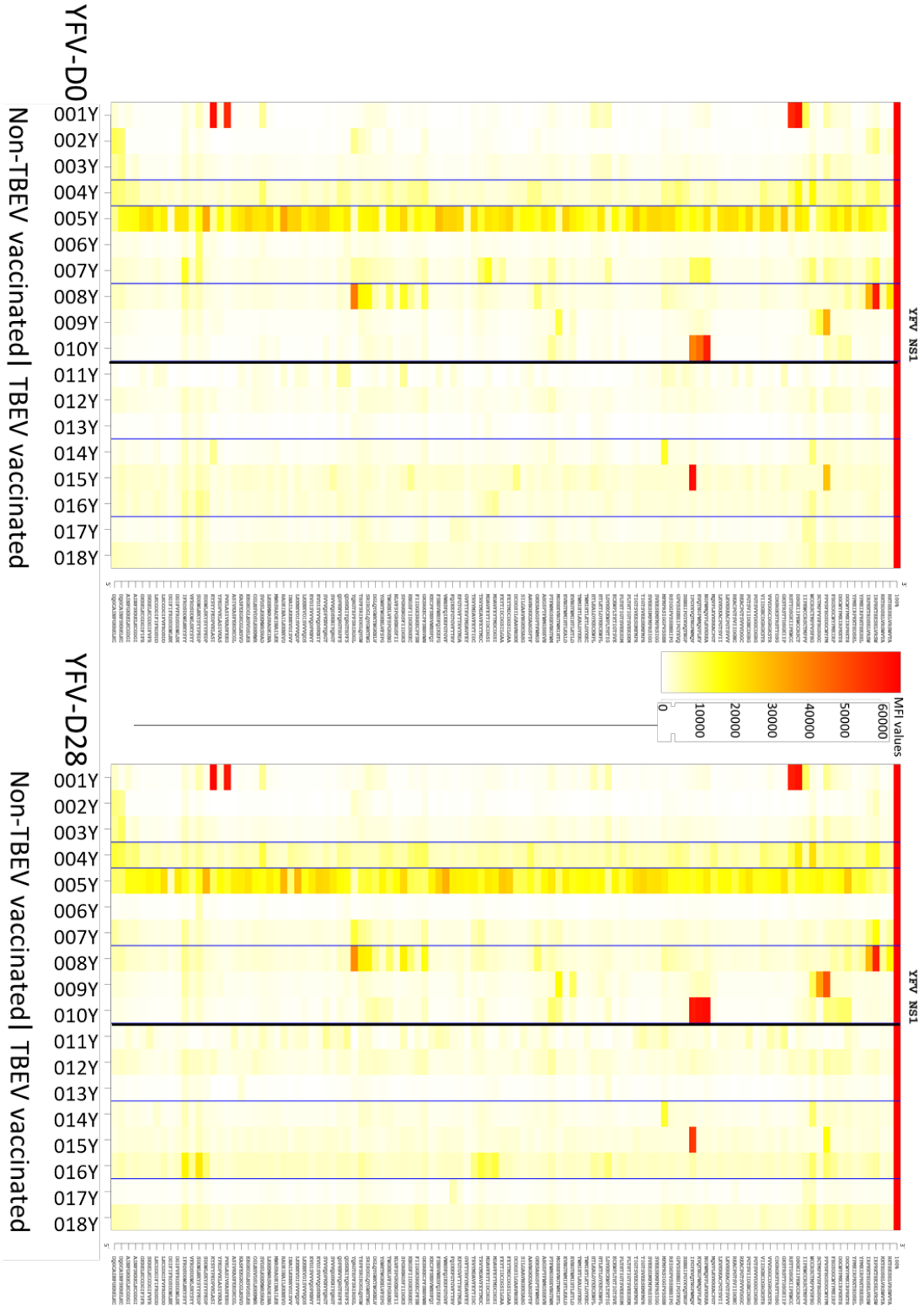

Figure S 7 Color-coded heat map of YFV-vaccination samples. Study subjects prior (YFV-D0, upper heat map) as our control group and after vaccination (YFV-D28, lower heat map) subdivided into study subjects with no history of TBEV vaccination (non-TBEV-vaccinated) and with history of TBEV vaccination (TBEV-vaccinated). Mean fluorescent intensities (MFI) of the YFV-vaccinated samples on x-axis plotted against all the different peptide sequences mimicking epitopes of the YFV E protein on y-axis. No visual difference in IgG epitope recognition across samples between two groups (YFV-D0 vs. YFV-D28) detectable. X-axis: study subject sera; y-axis: peptides along the sequence (N-terminus: bottom); legend to MFI values located in the center.

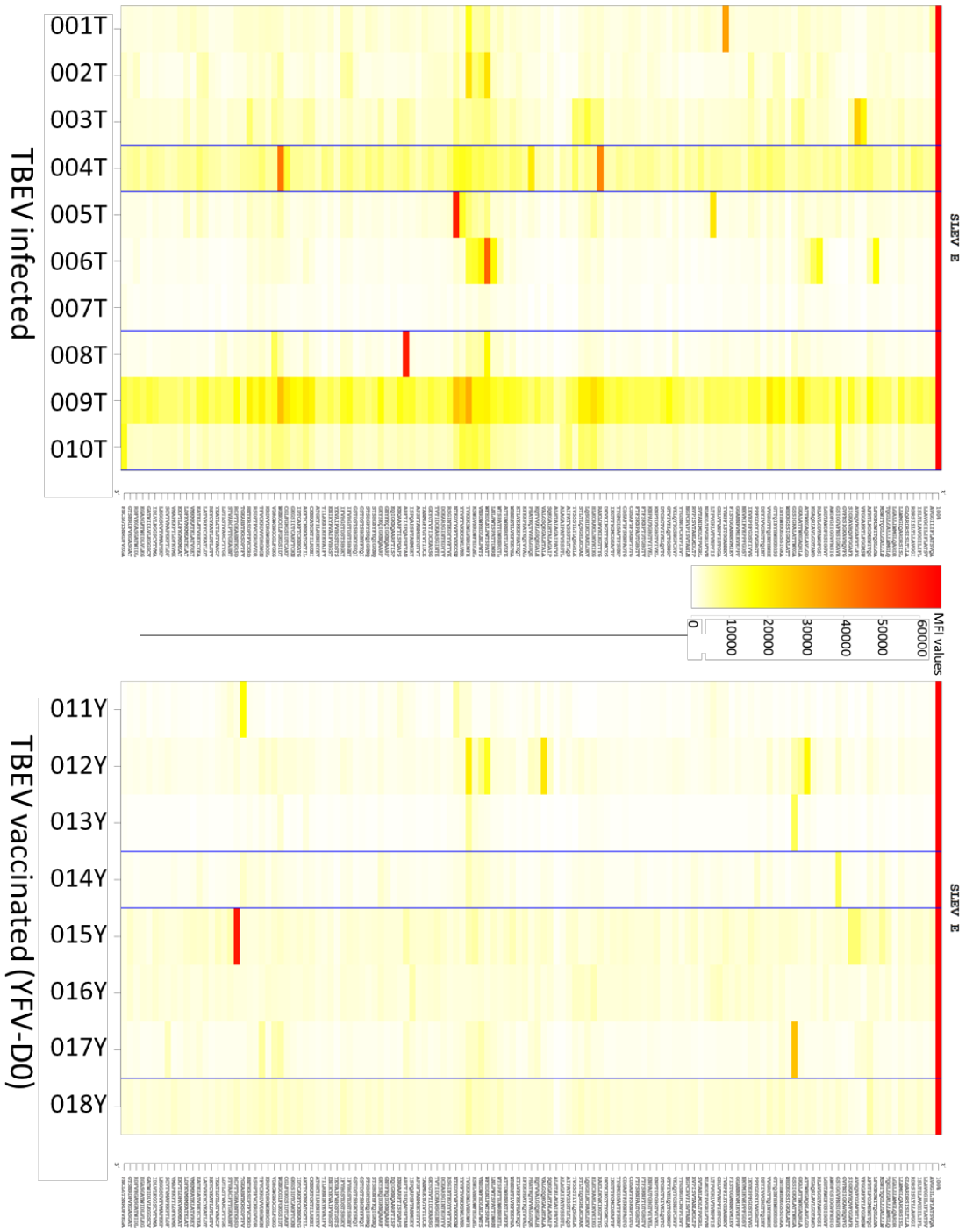

Figure S 8 Color-coded heat map of TBEV-infected (upper heat map) and TBEV-vaccinated study subjects (YFV-D0, lower heat map). Mean fluorescence intensities (MFI) of the TBEV samples on x-axis plotted against all different peptide sequences mimicking epitopes of the SLEV E protein on y-axis. TBEV antigens aren't displayed on the array, therefore the IgG responses are demonstrated for the related SLEV. A stronger IgG response in TBEV-infected individuals is elicited compared to vaccinated ones. X-axis: study subject sera; y-axis: peptides along the sequence (N-terminus: bottom); legend to MFI values located in the center.

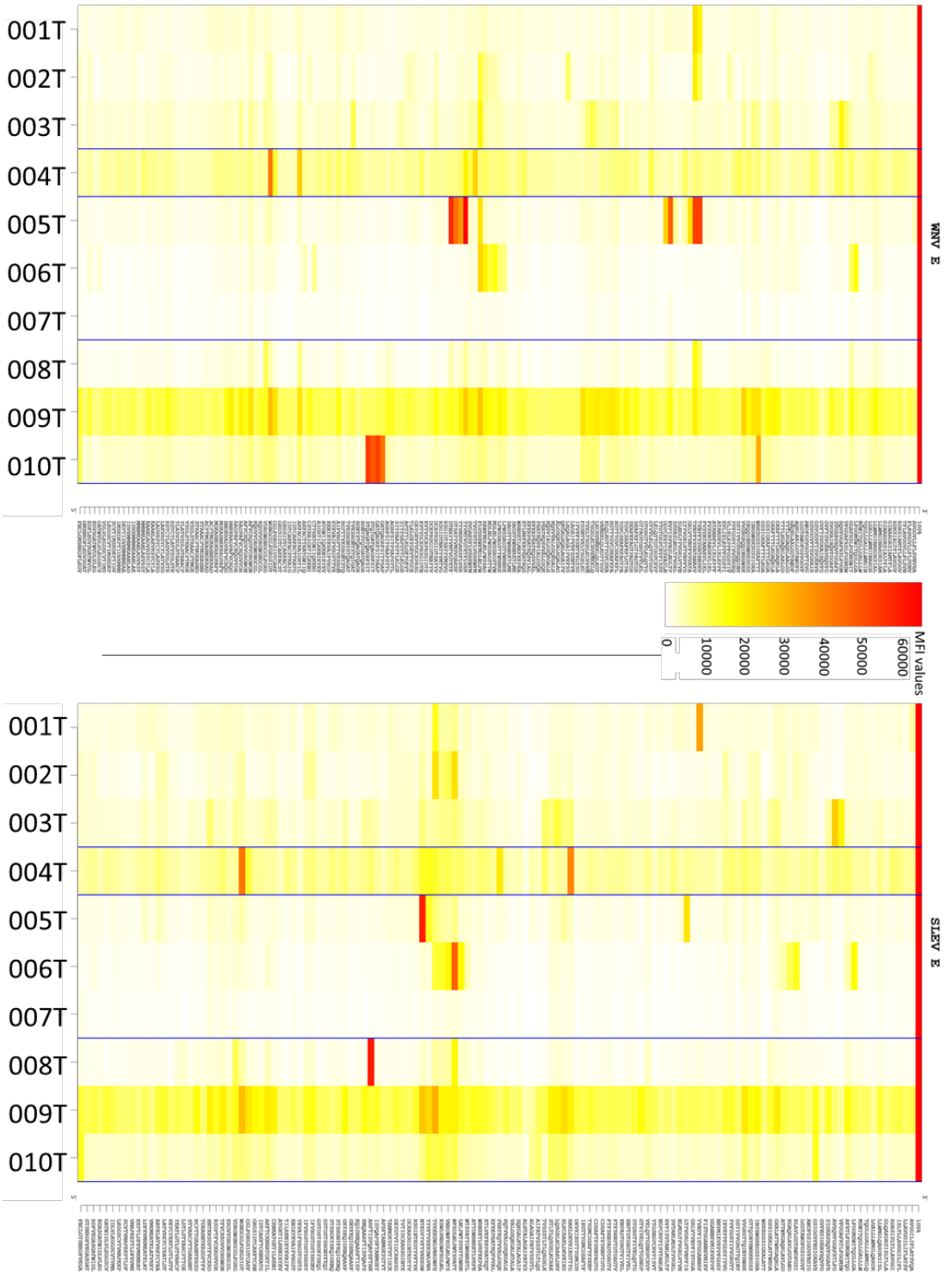

Figure S 9 Color-coded heat map of TBEV-infected study subjects. Mean fluorescent intensities (MFI) of TBEV samples on x-axis plotted against all the different peptide sequences mimicking epitopes of the WNV E protein (upper graph) and SLEV E protein (lower graph). Visual differences in IgG recognition pattern of WNV and SLEV epitopes. X-axis: study subject sera; y-axis: peptides along the sequence (N-terminus: bottom); legend to MFI values located in the center.

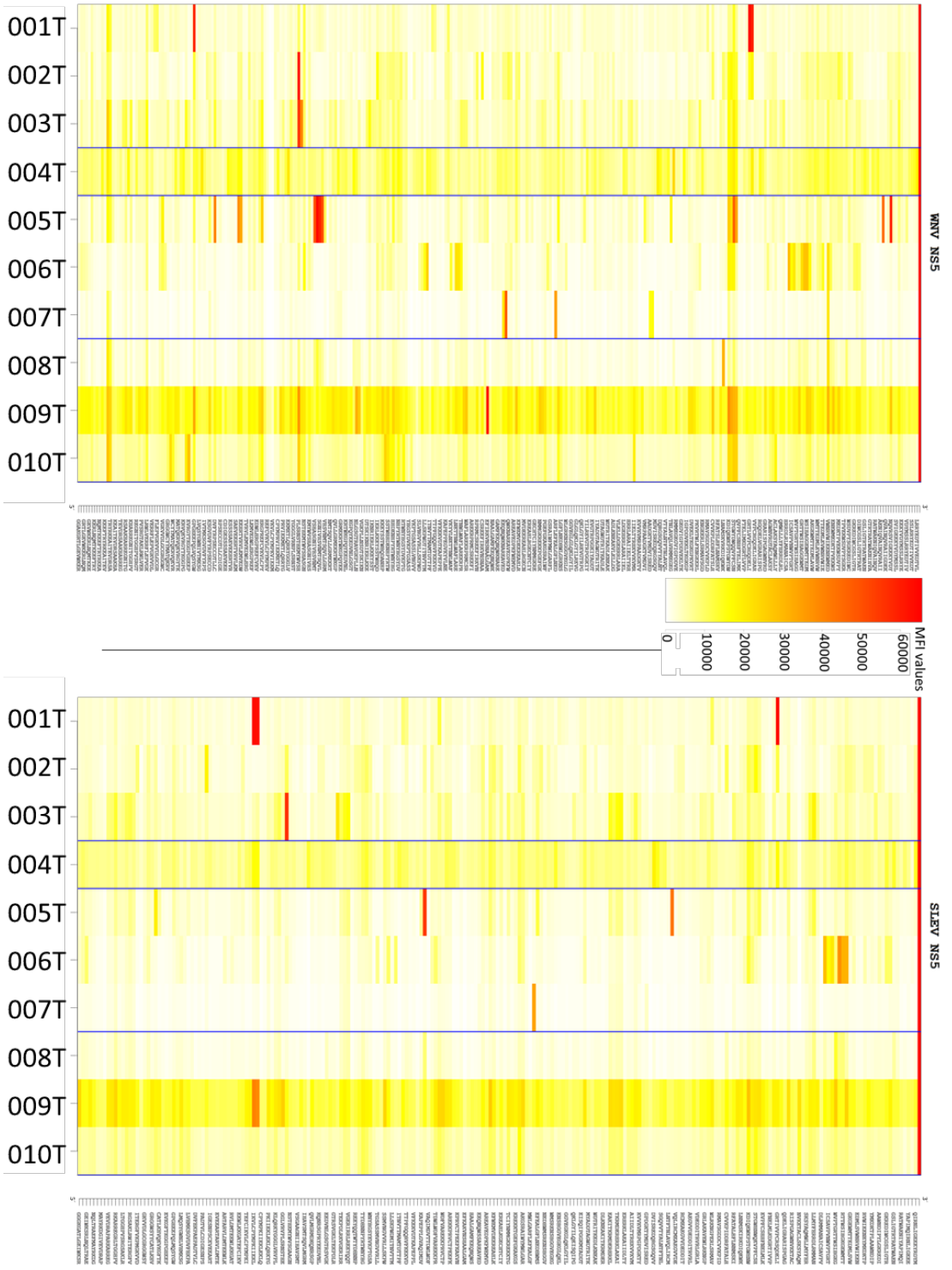

Figure S 10 Color-coded heat map of TBEV infection samples. Mean fluorescent intensities (MFI) of the TBEV samples on x-axis plotted against all the different peptide sequences mimicking epitopes of the WNV NS5 protein (upper graph) and SLEV NS5 protein (lower graph). Visual differences in IgG recognition pattern of WNV and SLEV NS5 epitopes as well as epitopes of E protein in WNV and SLEV across viruses and study subjects can be detected. Nevertheless, no epitope recognition pattern can be detected. X-axis: study subject sera; y-axis: peptides along the sequence (N-terminus: bottom); legend to MFI values located in the center.

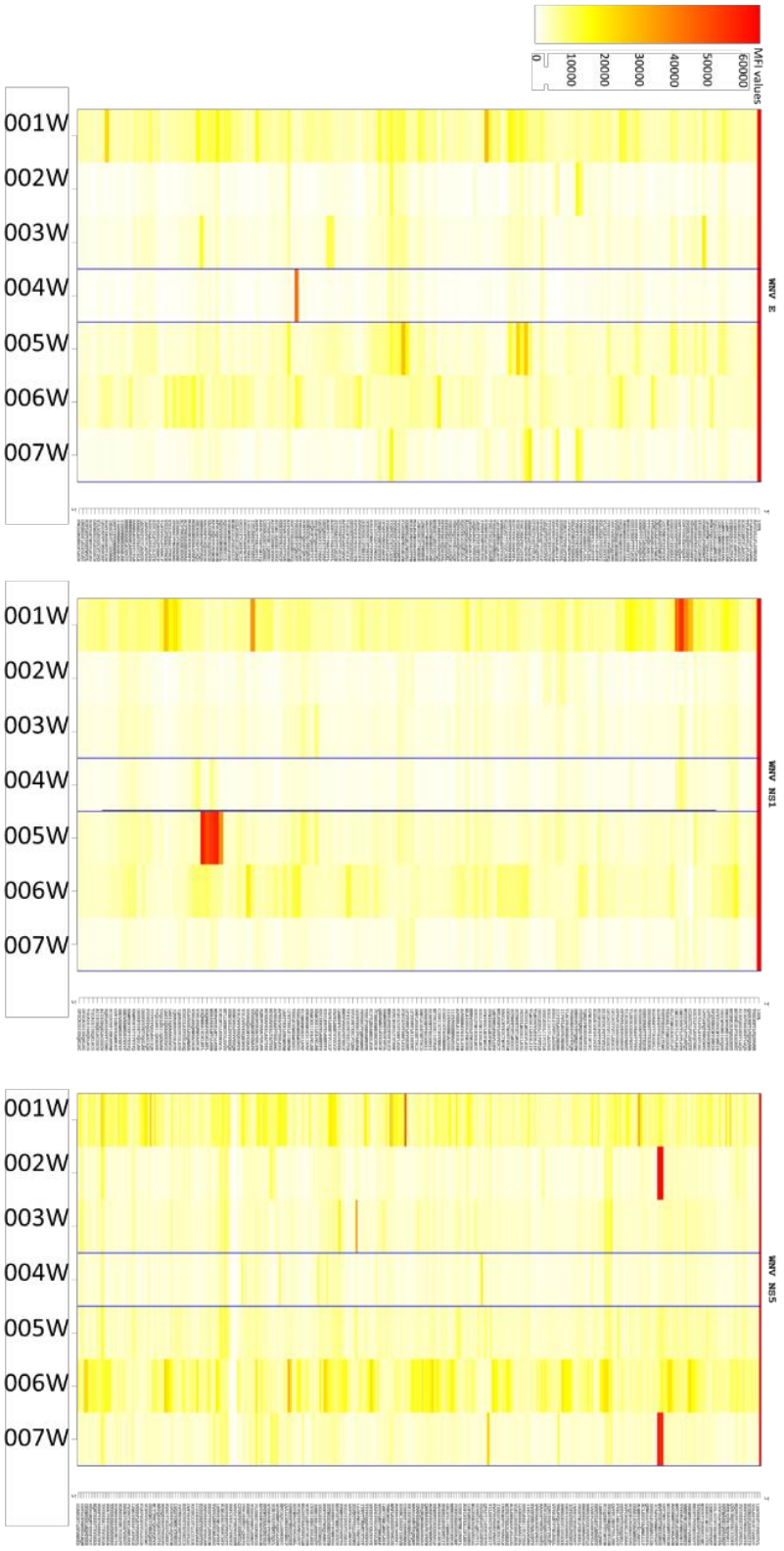

Figure S 11 Color-coded heat map of WNV infection samples. Mean fluorescent intensities (MFI) of the WNV samples on x-axis plotted against all the different peptide sequences mimicking epitopes of the WNV E protein (top graph), WNV NS1 protein (middle graph) and WNV NS5 protein (bottom graph). Compared to MFI values of TBEV-infected study subjects for WNV E and NS5 proteins [Figure S 10], most study subjects infected with WNV reacted weakly against autologous epitopes. X-axis: study subject sera; y-axis: peptides along the sequence (N-terminus: bottom); legend to MFI values located on the left.

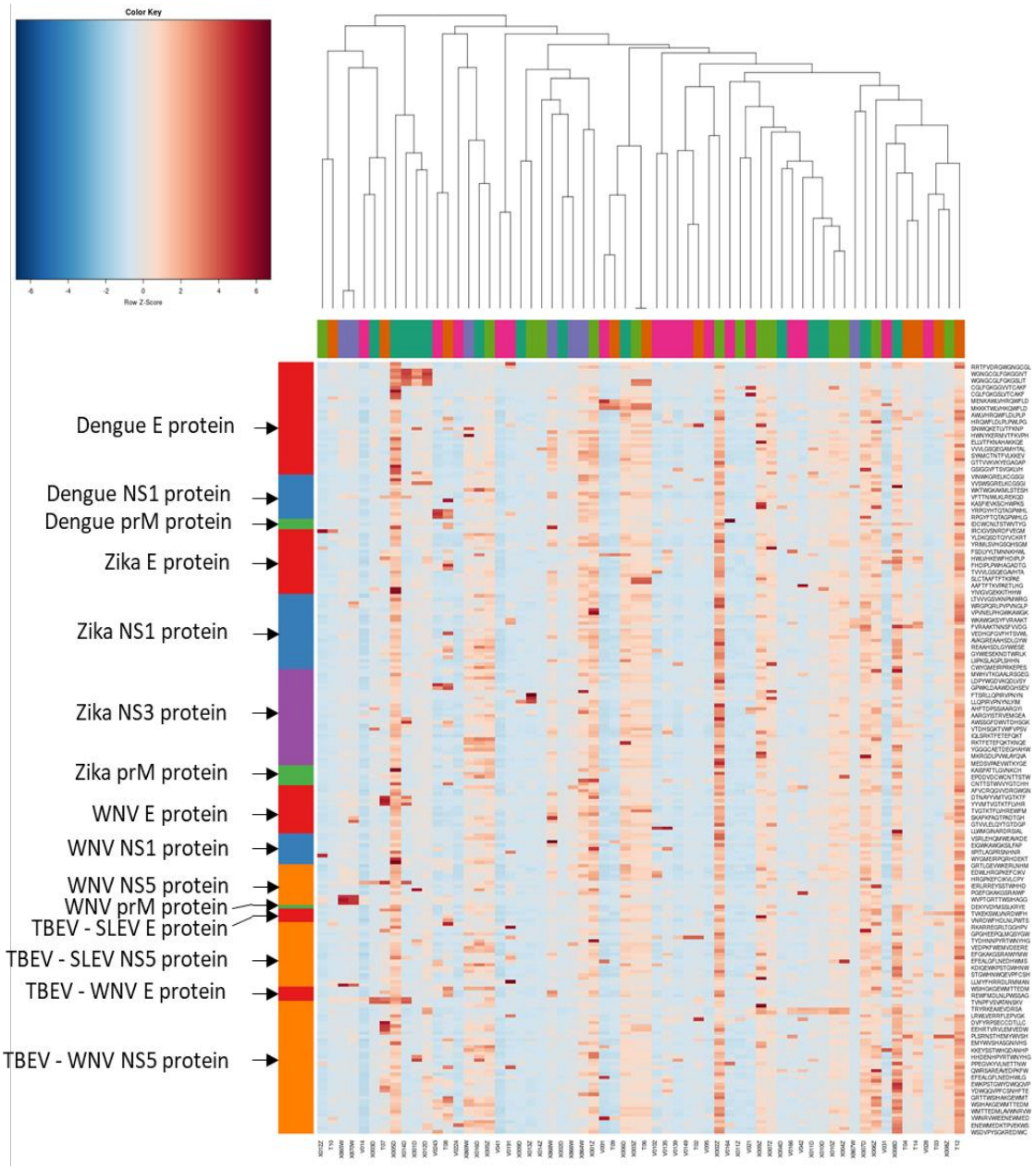

Figure S 12 Advanced heat map with pairwise alignment showing complete absence of immunodominant regions strongly recognized by all flavivirus-infected study subjects (within one flavivirus & across different flaviviruses). The ZIKV, DENV, TBEV and WNV infected as well as YFV D0 study subjects are intermingling displayed on the x-axis according to pairwise alignment of z-scores and plotted against the different epitopes of various proteins on the y-axis. X-axis: different flavivirus study subject samples; y-axis: peptides along the previously selected potential flavivirus-type-specific sequences (N-terminus: bottom) within different flavivirus proteins (sequences not shown in detail); z-score color key displayed on the top left.

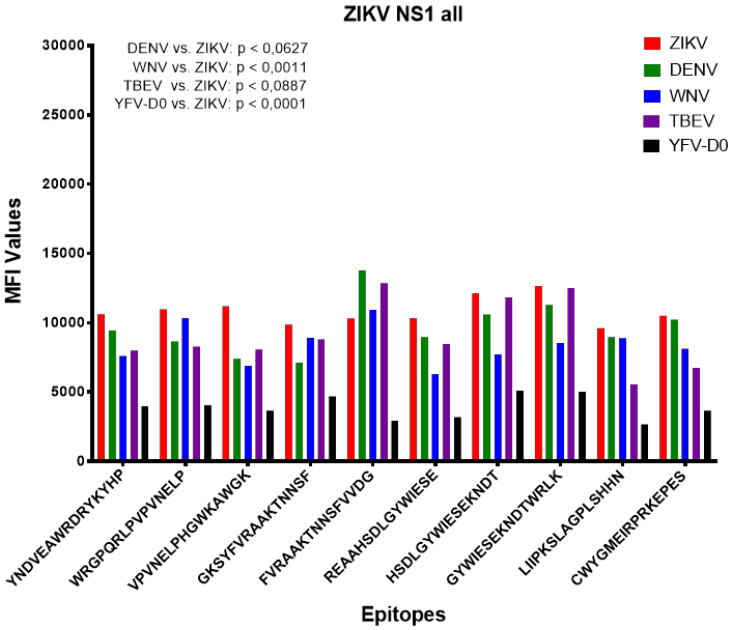

Figure S 13 Overview of epitope specificity for ZIKV NS1 protein of further selected epitopes. The selection is based on calculation of mean for each epitope higher than overall study subject mean [see chapter 3.5 statistics & graphing]. The epitopes were then plotted against mean IgG reactivity of other flavivirus study subjects and control group (YFV-D0) to determine flavivirus-specificity. According to p values, differences between ZIKV and WNV as well as YFV-D0 are considered significant within previously selected epitopes of ZIKV NS1 (p < 0.05). X-axis: different epitopes of protein; y-axis: MFI values; p values displayed in the graph.

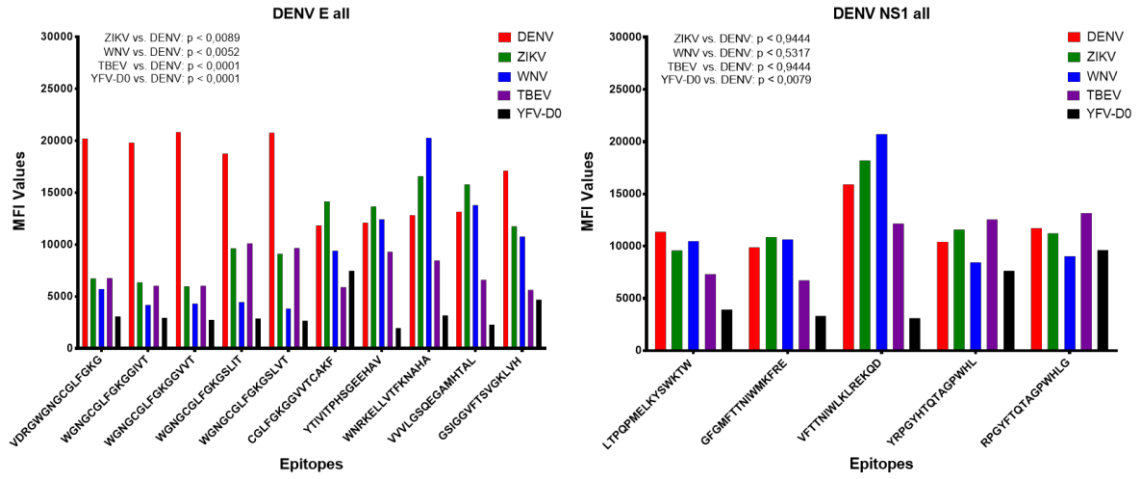

Figure S 14 Overview of epitope specificity for DENV E protein (left graph) and DENV NS1 protein (right graph) of further selected epitopes. The selection is based on calculation of mean for each epitope higher than overall study subject mean [see chapter 3.5 statistics & graphing]. The epitopes were then plotted against mean IgG reactivity of other flavivirus study subjects and control group (YFV-D0) to determine flavivirus-specificity. All p values for previously selected epitopes within DENV E protein are considered significant (p < 0.05)., whereas mostly non-significant results are obtained for DENV NS1 protein. X-axis: different epitopes of protein; y-axis: MFI values; p values displayed in the graph.

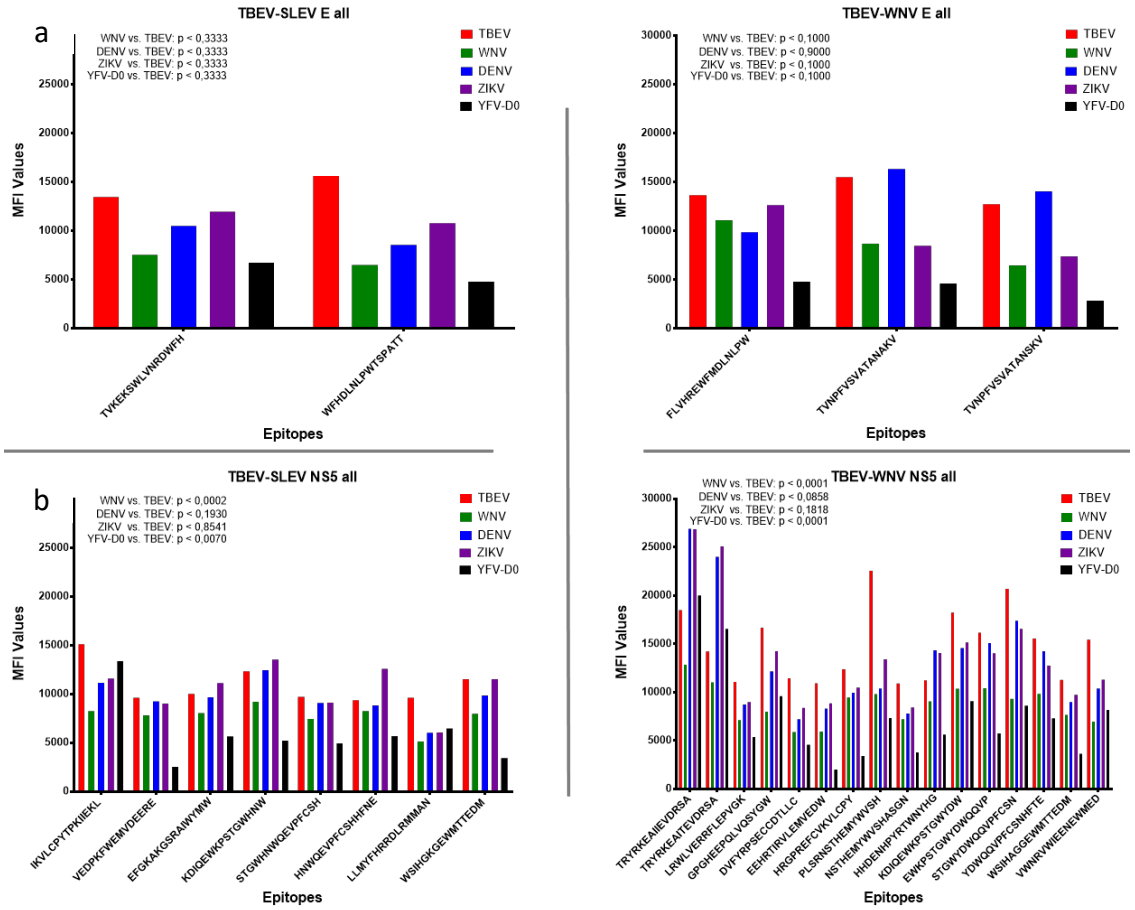

Figure S 15 Overview of epitope specificity for TBEV-SLEV E (a, left graph), TBEV-SLEV NS5 (a, right graph) as well as TBEV-WNV E (b, left graph) and TBEV-WNV NS5 (b, right graph) of further selected epitopes. Due to the fact, that TBEV epitopes are not displayed on the array, the IgG reactivity of TBEV-infected study subjects against the selected epitopes within E and NS5 protein for SLEV (TBEV-SLEV) and WNV (TBEV-WNV) were established. The selection is based on calculation of mean for each epitope higher than overall study subject mean [see chapter 3.5 statistics & graphing]. The epitopes were then plotted against mean IgG reactivity of other flavivirus study subjects and control group (YFV-D0) to determine flavivirus-specificity. Except for a few exceptions showing significant p values ($p < 0.05$), almost all p values are considered non-significant. X-axis: different epitopes of protein; y-axis: MFI values; p values displayed in the graph.

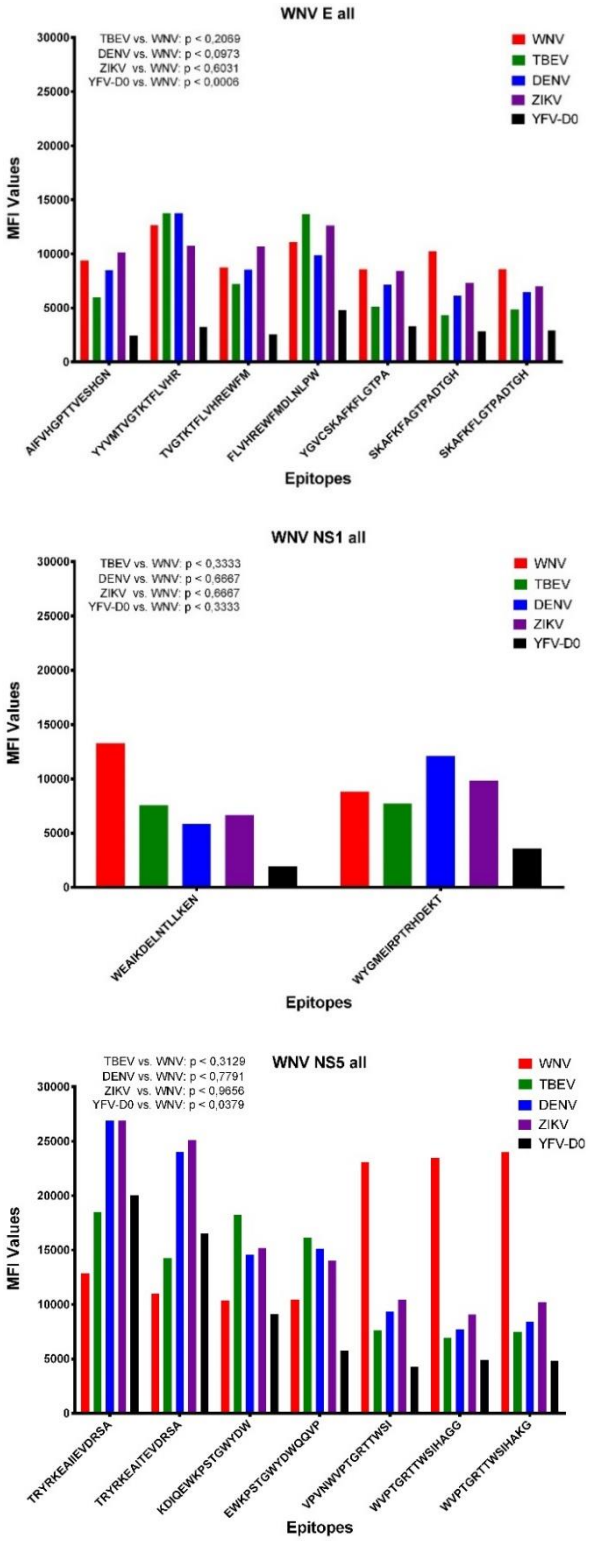

Figure S 16 Overview of epitope specificity for WNV E protein (top graph), WNV NS1 protein (middle graph) and WNV NS5 protein (bottom graph) of further selected epitopes. The selection is based on calculation of mean for each epitope higher than overall study subject mean [see chapter 3.5 statistics & graphing]. The epitopes were then plotted against mean IgG reactivity of other flavivirus study subjects and control group (YFV-D0) to determine flavivirus-specificity. Except for significant differences according to p-values ($p < 0.05$). comparing YFV-D0 and WNV study subject sera for previously selected epitopes in WNV E and NS5 protein, all other p values are considered non-significant. X-axis: different epitopes of protein; y-axis: MFI values; p values displayed in the graph.

Eidesstattliche Versicherung

Pieroth, Nora Simone

Name, Vorname

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Thema

Complete absence of linear immunodominant epitope regions recognized by IgG after flavivirus infection and vaccination in whole proteome analyses

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