universität wien

DISSERTATION

Fine-specificity and functional quality of the antibody response in mice to whole inactivated West Nile virus and recombinant subunit antigens

angestrebter akademischer Grad

Doktor/in der Naturwissenschaften (Dr. rer.nat.)

Verfasserin / Verfasser:	Mag. Jürgen Zlatkovic
Dissertationsgebiet (lt. Studienblatt):	Genetik-Mikrobiologie
Betreuerin / Betreuer:	O. Univ. Prof. Dr. Franz X. Heinz

Wien, im Dezember 2010

DANKSAGUNG

Diese Arbeit wurde von Oktober 2005 bis November 2010 am Department für Virologie der Medizinischen Universität Wien durchgeführt. An dieser Stelle gilt mein Dank all jenen ohne deren Einsatz und Hilfe die Dissertation in dieser Form nicht möglich gewesen wäre.

Mein besonderer Dank gilt Prof. Franz X. Heinz der mir die Möglichkeit und Mittel gab um diesen wichtigen Abschnitt meiner Ausbildung am Department für Virologie durchzuführen. Im Speziellen möchte ich mich für die wissenschaftliche Anleitung und die ständige Bereitschaft für konstruktive und kritische Diskussionen bedanken.

Weiters möchte ich mich ganz herzlich bei Karin Stiasny für die Hilfestellung bei allen wissenschaftlichen Fragestellungen, vor allem was Testetablierung und die Auswertung und Interpretation von Daten anging, bedanken.

Stefan Kiermayr danke ich für die Einführung in die Laborgepflogenheiten am Institut und für das Erlernen des technischen und theoretischen Basiswissens zur Produktion rekombinanter Proteine in den verschiedenen Systemen.

Oksana Vratskikh und Karen Pangerl für die notwendige Hilfe und Unterstützung bei den Tierversuchen.

Allen Mitarbeitern des Departments für Virologie für ihre Hilfe und das freundliche Arbeitsklima.

Diese Arbeit möchte ich all jenen widmen die mich unterstützt haben, Familie wie auch Freunde, welche in guten und schlechten Zeiten immer motivierende Worte gefunden haben und mir mit Rat und Tat beiseite gestanden sind.

Contents

SUMMARY

Flaviviruses form a genus in the family of *Flaviviridae* and comprise a number of important human pathogens, such as yellow fever, dengue, West Nile, Japanese encephalitis, and tick-borne encephalitis viruses. These viruses have a world wide distribution and the potential to emerge in new geographic areas and thus are a major concern for public health. Structurally, flaviviruses are extremely well characterized by a combination of cryo-electron microscopy and X-ray crystallography of the major envelope protein E. Infection or immunization with flaviviruses induces a protective antibody response which is mainly directed to E. Epitopes involved in virus neutralization were identified in all domains of E, having different neutralizing potential. Broadly cross-reactive antibodies, directed to the so-called fusion loop in E, appear to have relatively low neutralizing activity but comprise a major fraction in human post-infection sera. In contrast, antibodies to domain III (DIII) of E have the highest neutralizing potency and represent a significant fraction in murine but not in human post-infection sera.

The major objective of this thesis was to investigate the influence of the physical organisation of flavivirus immunogens on the fine-specificity, immunodominance and functional quality of antibody responses. It was a further objective to specifically design prime-boost immunization regimens that focus the antibody response to the highly neutralizing sites of DIII in E. For these purposes, mouse immunization studies were conducted using whole inactivated purified virus (displaying a closed shell of tightly packed E proteins), a monomeric soluble form of E and the isolated DIII of West Nile virus as immunogens.The major findings of this work have been published in the *Journal of Virology* (*Part I* of the thesis).

Analyses of the post-immunization sera revealed that virion and sE induced significantly different proportions of DIII-reactive antibodies which in both cases dominated the neutralizing response. However, the functional quality of the DIII-reactive antibodies induced by virion immunization was 4 times higher than that after sE immunization, and those induced by the isolated DIII had an even 15-fold lower neutralizing quality compared to the virion. They also failed to completely neutralize the virus and only 50% of these antibodies were able to bind to viral particles. These results suggest that the E protein in its tightly packed arrangement on the surface of whole virions is an extremely efficient form presenting those DIII epitopes that are

primary targets for neutralization. In contrast, soluble antigens focus the antibody response not as efficiently to the determinants critical for neutralization but rather induce antibodies to sites not exposed on the viral surface and thus contribute less to neutralization. Further analyses revealed that the avidity of the antibodies for the virus were highest after virion- followed by sE- and DIII-immunization, representing a possible additional contributing factor to the distinct neutralizing quality of the DIII-reactive antibodies.

Sequential prime-boost immunization regimens using DIII either for priming or for boosting demonstrated that DIII is an excellent booster antigen which restimulates the DIII specific neutralizing response after priming with virion or sE more strongly than the homologous antigen. Surprisingly, priming with DIII followed by sE or virion immunization resulted in a dampening of the antibody response compared to mice without DIII priming.

The data presented provide novel insights into the fine-specificity of the antibody response induced by virus as compared to recombinant subunit vaccines and demonstrated the potential of using an individual protein domain (DIII) for boosting the most strongly virus neutralizing antibodies. These findings have implications for the specific design of future vaccines and vaccination regimens.

ZUSAMMENFASSUNG

Flaviviren bilden ein Genus in der Familie *Flaviviridae* und umfassen eine Reihe von wichtigen Krankheitserregern wie Gelbfieber, Dengue, Japanische Enzephalitis, Frühsommer-Meningoenzephalitis oder West Nil Viren. Aufgrund ihrer weltweiten Verbreitung und dem möglichen Auftreten in neuen geographischen Regionen repräsentieren Flaviviren Krankheitserreger mit hoher epidemiologischer und klinischer Relevanz. Die Struktur von Flaviviren wurde mit Hilfe von Kryo-Elektronenmikroskopie und Kristallographie des viralen Hauptoberflächenproteins E aufgeklärt. Die nach Infektion oder Immunisierung mit Flavivirus induzierten Antikörper sind protektiv und zum größten Teil gegen E gerichtet. Epitope neutralisierender Antikörper wurden in allen Domänen des E Proteins identifiziert. Breit Flavivirus kreuzreaktive Antikörper, welche an das Fusionspeptid in E binden, haben zwar meist relativ geringe neutralisierende Aktivität, repräsentieren aber generell einen signifikant hohen Anteil in humanen Post-Infektionsseren. Domäne III (DIII)-spezifische Antikörper haben die stärkste neutralisierende Aktivität und bilden in Mäusen einen bedeutenden Anteil an der E Protein spezifischen Antikörperantwort. Im Gegensatz dazu, dominiert beim Menschen die Antikörperantwort gegen das Fusionspeptid.

Das Hauptziel dieser Dissertation bestand darin zu erforschen, ob der strukturelle Kontext des Immunogens die Feinspezifität, Immundominanz und funktionelle Qualität von Antikörperantworten beeinflusst. Weiters wurde untersucht, ob es möglich ist, mit sequentiellen Immunisierungen, unter Verwendung der isolierten DIII, die Antikörperantwort auf Epitope stark neutralisierender Antikörper in DIII zu fokussieren. Aus diesem Grund wurde eine Immunisierungsstudie im West Nil Virus System durchgeführt, bei der Mäuse mit Formalin inaktiviertem, gereinigtem Virus (umhüllt von einem kompakten Mantel aus dicht gepackten E Proteinen), dem löslichen E Protein (Monomer) und löslicher isolierter DIII, immunisiert wurden. Die Hauptergebnisse dieser Arbeit wurden im *Journal of Virology* veröffentlicht (siehe *Part I* der Dissertation).

Die Analyse der Maus-Immunseren zeigte, dass sE einen signifikant höheren Anteil an DIII spezifischen Antikörpern induziert als das Virus, wobei diese in beiden Fällen den Großteil der neutralisierenden Antikörper ausmachten. Die spezifische neutralisierende Qualität der DIII Antikörper war nach Immunisierung mit Virus jedoch

4-fach höher. Die isolierte DIII induzierte nur eine vergleichbar geringe neutralisierende Antwort. Die spezifische neutralisierende Qualität dieser Antikörper war, verglichen mit Virus, zumindest 15-fach geringer, und 50% dieser Antikörper konnten in Absorptionsexperimenten nicht an das Viruspartikel binden. Diese Daten deuten darauf hin, dass das E Protein in seiner dicht gepackten Form an der Oberfläche viraler Partikel hauptsächlich jene Epitope in DIII präsentiert, die Ziele neutralisierender Antikörper sind. Im Gegensatz dazu präsentieren lösliche Antigene Epitope, die an der Oberfläche des viralen Partikels nicht zugänglich sind und somit teilweise Antikörper induzieren die nicht oder weniger zur Neutralisation von infektiösem Virus beitragen.

In sequentiellen Immunisierungen mit DIII zeigte sich, dass durch Verabreichung von DIII nach Vorimmunisierung mit Virus oder sE die neutralisierende Antwort, im Vergleich zur Immunisierung mit den homologen Antigenen (sE oder Virus), deutlich gesteigert werden konnte. Wurde DIII jedoch als Grundimmunisierung vor Virus oder sE administriert, wurde eine Reduktion der Antikörperantwort, im Vergleich zu Mäusen ohne Vorimmunisierung, beobachtet.

Diese Arbeit liefert neue Erkenntnisse über die Feinspezifität der Antikörperantwort in Mäusen nach Immunisierung mit inaktiviertem Virus und rekombinanten löslichen Antigenen und eröffnet die Möglichkeit mit Hilfe von individuellen, isolierten Domänen die neutralisierende Antwort gegen das Virus zu steigern. Damit können die vorgelegten Ergebnisse zur Entwicklung neuer Immunisierungsstrategien beitragen.

1. Introduction

1.1 Flaviviruses – taxonomy and distribution

Flaviviruses form a genus in the family of *Flaviviridae*, consisting of more than 70 different viruses (25). According to their antigenic relationship, flaviviruses are grouped into so-called serocomplexes (Fig. 1.1) (8). Important human pathogenic representatives are dengue (DEN), Yellow fever (YF), West Nile (WN), Japanese encephalitits (JE) and tick-borne encephalitis (TBE) viruses (Fig. 1.1). They are distributed world wide and represent a major concern for public health in tropical and subtropical regions of the Americas (DEN, WN, YF), Africa (DEN, WN, YF) and Asia (DEN, JE, TBE), as well as in Europe (WN, TBE). Flaviviruses are mainly transmitted by vectors like mosquitoes or ticks, however for some representatives like Modoc or Rio Bravo viruses, no vectors have been described yet.

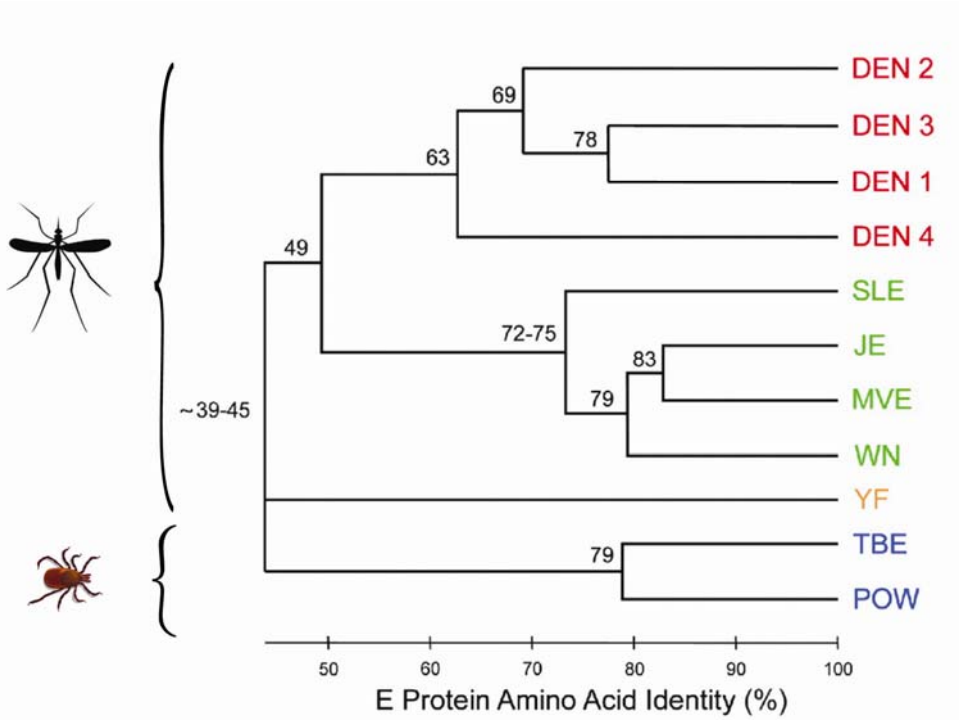

Fig 1.1 Dendrogram displaying antigenic serocomplexes of flaviviruses.
Distance relationships of different flaviviruses are indicated by the E protein amino acid identity in %. The serocomplexes are shown in red for dengue, green for Japanese encephalitis, orange for Yellow fever and blue for the tick-borne encephalitis virus serocomplex. Figure adapted from (94).

1.2 West Nile virus

1.2.1 Taxonomy and epidemiology

West Nile virus is a mosquito-borne flavivirus in the Japanese Encephalitis virus serocomplex (Fig. 1.1) and represents one of the most widely distributed flaviviruses world-wide (31). According to nucleotide sequence analysis, West Nile viruses can be divided into two genetically distinct lineages (54). Lineage I viruses, emerging globally, are distributed in Mediterranean parts of Africa and Europe, West and Central Asia, the Middle East, the Americas and Australia. Lineage II viruses are predominantly found in sub-Saharan parts of Africa but have also been recently detected in Central Europe (3).

West Nile viruses are important animal and human pathogens causing generally asymptomatic or mild febrile diseases but, in some cases, severe potentially fatal disease forms including encephalitis and meningitis. The virus was first isolated in 1937 from a febrile patient in the West Nile district of Uganda (91). Until the 1990s, sporadic epidemics with few severe fatal cases were reported in Africa, Russia and Israel, mostly in rural populations. In the 1990s, epidemics became more frequent as exemplified by larger outbreaks, also in urban populations, for instance in Romania (1996) and Russia (1999). In summer 1999, natural human infections were reported for the first time in North America during an outbreak in the city of New York (55). Sequence studies suggested that the virus, belonging to lineage I viruses, was most likely introduced from Israel into the United States (29).

Until 2004, lineage II strains were detected in sub-Saharan parts of Africa only. However, in 2004, a lineage II strain WN virus was detected for the first time in Central Europe (3) in infected animals. In addition, human infections including fatal cases caused by lineage II viruses were reported in Russia, Romania and recently in Greece in 2010 (73).

The appearance and spread of WN virus in the Americas and the emergence of lineage II virus strains in Europe and Russia demonstrated the potential of West Nile virus to emerge in new geographical areas.

1.2.2 Transmission

West Nile virus is maintained in nature in a mosquito-bird transmission cycle involving predominantly mosquitoes of the genus Culex. More than 100 different bird species like sparrows, crows and grackles, which serve as the virus-amplifying hosts, can get infected with West Nile virus. After infection, birds develop a high level of viremia and are either asymptomatic or develop potentially fatal disease. Mosquitoes can get infected by having a blood meal on birds with a sufficiently high virus titer. In the mosquito, the virus replicates in gut epithelium and disseminates to tissues including the salivary glands for further amplification.

Humans or other mammals like horses and small rodents can also get infected with WN virus. They develop only moderate levels of viremia and, most likely, do not contribute to the transmission cycle and are therefore considered as dead end hosts. However, they can develop mild or severe diseases, including fatal forms. Human-to-human transmission has been documented sporadically by blood transfusions, organ transplants or breast feeding from viremic donors to non-immune recipients (35).

1.2.3 Pathogenesis

1.2.3.1 Clinical manifestations in humans

Seroprevalence studies in humans suggest that 80% of the West Nile virus infected individuals do not develop any significant disease symptoms. In clinically overt cases, febrile, flu-like symptoms occur within 2-14 days post-infection. The disease is generally self limiting, however, about 1% of the patients develop severe neuroinvasive forms like meningitis, encephalitis, or acute flaccid paralysis with a mortality rate of 10-20% (9). Immunosuppressed individuals like transplant patients, HIV-infected or elderly, represent groups which have an increased risk for developing severe disease forms.

1.2.3.2 Viral dissemination in humans

After inoculation through mosquitoes, WN virus infects and replicates in skin Langerhans dendritic cells, which migrate to draining lymph nodes. The virus subsequently enters the blood stream via the thoracic duct or efferent lymphatic vessels causing a primary viremia. The virus continues to spread via the circulation and infects peripheral tissues like cells of the reticuloendothelial system and visceral organs, for instance spleen and kidney, leading to secondary viremia. West Nile virus is capable of crossing the blood brain barrier via the hematogenous route (20), retrograde axonal transport (85), or infected inflammatory cells. In the central nervous system apoptosis can be induced in infected neurons causing significant neuronal tissue damage.

1.3 Molecular biology of flaviviruses

1.3.1 Genome structure

The genome of flaviviruses is a ~11kb long, single stranded, positive sense RNA molecule. A cap structure is located at the 5` terminus, however, there is no poly-A tail at the 3`terminus. The non-coding regions (NCR) at the 5`and 3` terminal regions are highly structured and are involved in genome cyclization, replication and translation (63). The genome has a single open reading frame encoding three structural proteins C, prM, E and seven non-structural proteins NS1, 2A, 2B, 3, 4A, 4B, 5 (Fig. 1.2A), (60).

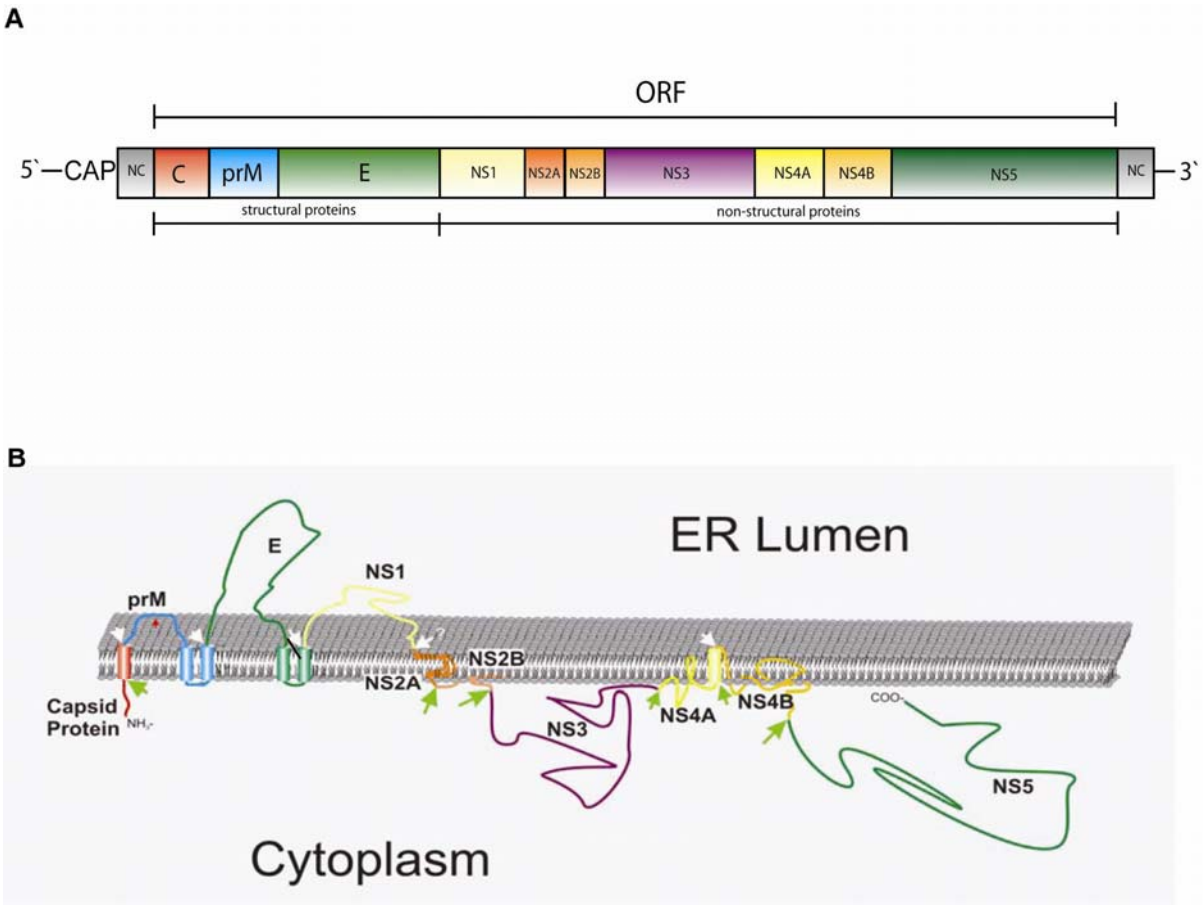

Fig. 1.2 Schematic representations of the flavivirus genome and polyprotein.
(A) A single long open reading frame encodes all structural proteins (C, prM, E) and non-structural proteins (NS1, 2A, 2B, 3, 4A, 4B, 5). NC indicates the non-coding regions at both ends of the genome. (B) Schematic of the polyprotein, and its association with the membrane of the endoplasmic reticulum (ER). Cleavage sites for proteolytic processing by viral and host proteases are indicated by green (NS2B3 cleavage sites), white (signalase cleavage sites) and red arrows (furin cleavage site). The figure was adapted from the PhD Thesis, K. Orlinger: 'Construction and Application of Bicistronic Flaviviruses'; University of Vienna 2007.

1.3.2 Structure of the viral particle

Flavivirus virions are small particles with a diameter of about 50 nanometres (nm). The single stranded positive sense RNA genome, which is about 11 kilobases (kb) long, is packed into a spherical capsid structure composed of the viral structural protein C. This nucleocapsid is enveloped by a host cell-derived lipid bilayer in which the viral transmembrane proteins E and prM/M are anchored (60).

In the host cell, flavivirus virions are assembled as immature particles, which are not infectious. The surface of such fully immature particles is covered by 60 trimers of prM-E heterodimers, forming spiky projections from the surface (Fig. 1.3 and 1.5C). During the passage of the virus through the Trans-Golgi Network (TGN) of the host cell, pH dependent structural rearrangements of E and the proteolytic digest of the prM protein into 'M' and 'pr', which dissociates from the virion at neutral pH in the extracellular milieu (57, 109), lead to a structural reorganization of the proteins on the viral surface. In fully mature viral particles, which are now infectious, the E proteins form 90 antiparallel, energetically metastable, homodimers, which are organized in a herringbone-like icosahedral lattice. They are oriented parallel to the viral membrane forming a smooth surface (Fig. 1.3 and 1.5D).

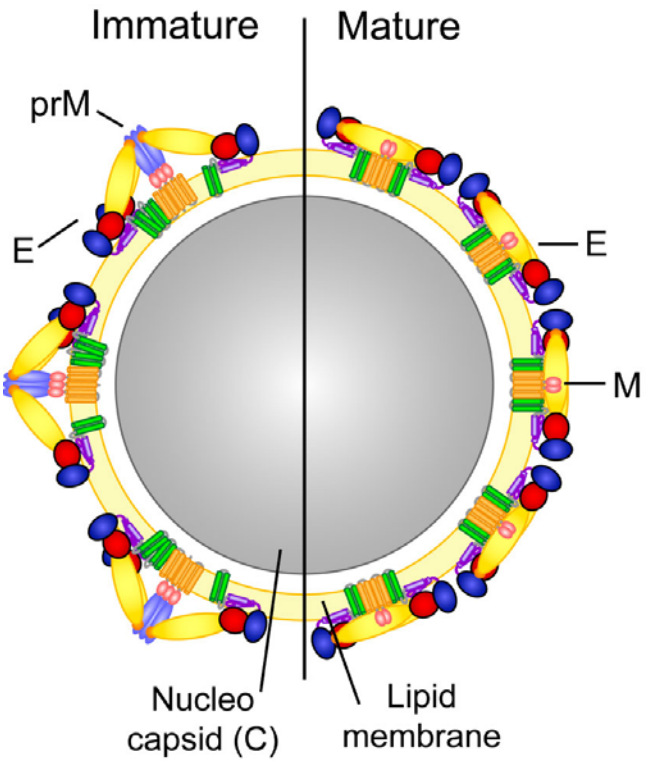

Fig. 1.3 Immature and mature flaviviruses.

Schematic representation of the prM-E heterodimers and E homodimers, associated with the lipid envelope, on the surface of immature and mature viruses, respectively. Individual parts of the E protein are shown in red for DI, yellow for DII, blue for DIII, followed by the stem and transmembrane regions indicated in purple and green, respectively. The pr peptide of prM is indicated in grey and the extracellular part of the M protein and the transmembrane domains are shown in pink and orange, respectively.

1.3.3 The flavivirus life cycle

Essential steps of the life cycle of flaviviruses are schematically shown in Fig. 1.4. The virus attaches to the cell via the E protein to poorly defined cellular receptors and is subsequently taken up by receptor-mediated endocytosis. In the endosome, acidic pH triggers structural rearrangements in the E protein, mediating fusion of the viral and endosomal membrane. The nucleocapsid is released into the cytoplasm, the RNA genome gets separated from the capsid protein (uncoating) and viral protein expression and genome replication is initiated. Flavivirus proteins are translated as a single polyprotein precursor from the genomic RNA and associate with the membrane of the endoplasmic reticulum (ER) (Fig. 1.2B). Individual proteins are separated co- and post-translationally by proteolytic processing of the polyprotein precoursor, by host signal peptidase in the lumen (signalase) and the viral serine protease (NS2B3) at the cytoplasmic side of the ER membrane (Fig. 1.2B). The structural proteins E and prM are translated into the lumen of the ER whereas the C protein is located at the cytoplasmic side (Fig 1.2B). Assembly of the viral particles takes place in the ER. The immature non-infectious particles are transported through the secretory pathway. In the late TGN, acidic pH triggers a conformational change in the prM protein rendering the furin cleavage site accessible. The prM protein is subsequently cleaved by furin into the “M” protein which remains associated with the particle via its transmembrane domains and the “pr” peptide which dissociates from the viral surface at neutral pH after secretion of the mature viral particle (57, 109). In addition to viral particles, subviral particles (slowly sedimenting hemagglutinin-SHA) are also produced upon natural infection. Subviral particles lack a nucleocapsid, are smaller than the virion, (diameter of approximately 30nm), and the mature secreted forms contain only 30 E protein dimers on the surface. They can also be produced in a recombinant form as so-called recombinant subviral particles (RSPs) by transfection of mammalian cells with a prM-E encoding DNA expression vector (2).

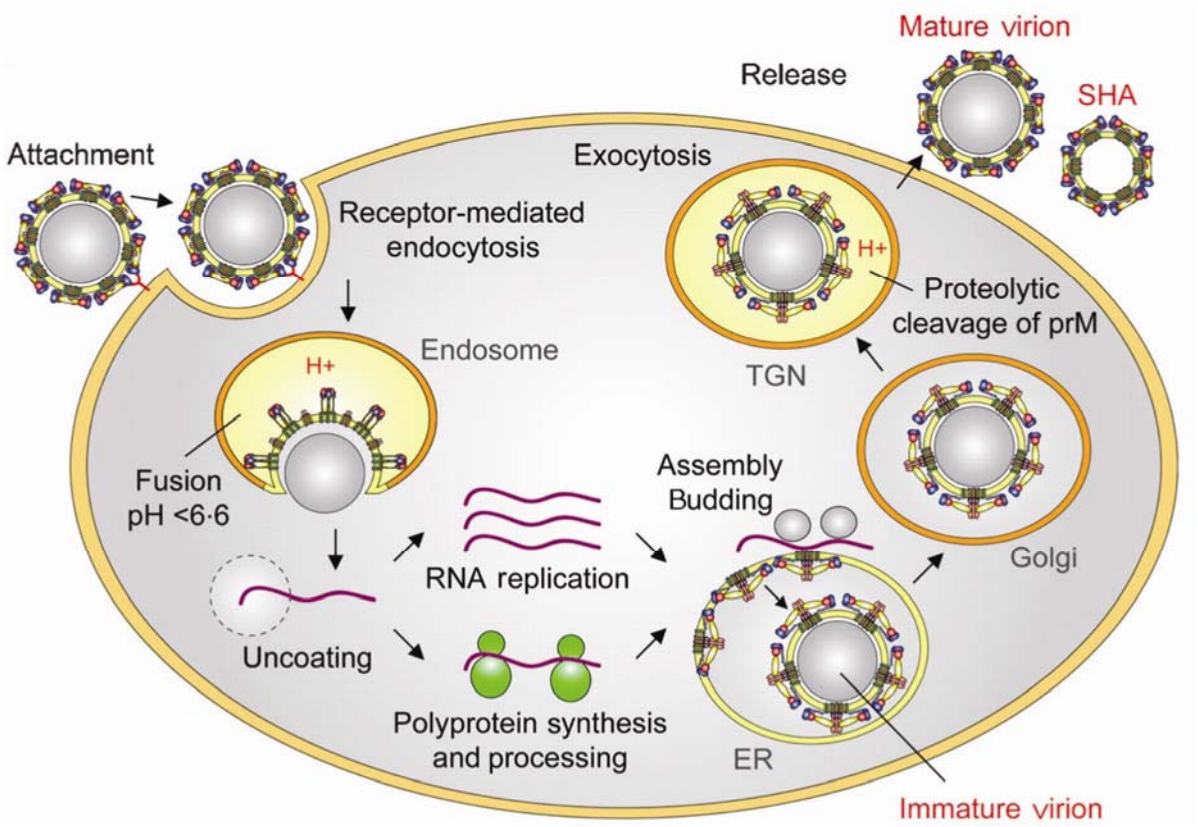

Fig. 1.4 Schematic representation of the flavivirus life cycle

Sequential steps of infection, replication, assembly, maturation and release are indicated. ER, endoplasmic reticulum; TGN, trans-Golgi network; SHA, slowly sedimenting hemagglutinin representing nucleocapsidless subviral particles. Figure adapted from (93).

1.3.4 Viral structural proteins

1.3.4.1 Surface glycoprotein E

The E protein, the major constituent of the virion surface, serves as the ligand for the attachment of the virus to cellular receptors and mediates fusion of the cellular and viral membrane in the endosome after receptor-mediated endocytosis of the virus. Because of these functions, the E protein is the major inducer of a neutralizing and protective antibody response (for further details see section 1.4).

It consists of approximately 500 amino acids and has a molecular mass of ~53 kilo Daltons (kDa). E is anchored in the viral membrane by two transmembrane alpha helices followed by the hydrophobic membrane proximal ‘stem’ region (Fig. 1.5B). The stem consists of two hydrophobic alpha helices which lie parallel to the

membrane and link the transmembrane parts and the ectodomain (110) (Fig. 1.5B). Crystal structures of soluble forms of the E protein (sE) from TBE, WN and DEN viruses, lacking the hydrophobic C-terminal stem and transmembrane regions, show that the E protein is organized into three distinct structural domains: domain I (DI) domain II (DII) and domain III (DIII) (45, 64, 65, 68, 79) (Fig 1.5A). DI is the central domain and is flanked by DII and DIII. It contains the glycosylation site(s) and two disulfide bridges and is mainly composed of ß-sheets and random coils (Fig 1.5A). DII is a finger-like structure, formed by two elongated loops emanating from DI, containing three disulfide bonds and, in addition to ß-sheets and random coils, also a short alpha helix. At its tip, DII contains the fusion peptide (FP) loop (Fig 1.5A), which is highly conserved among flaviviruses and an important functional element in the early stages of viral and endosomal membrane fusion (Fig. 1.4). Upon exposure to acidic pH in the endosome, the FP loop gets exposed and makes contact with the endosomal membrane thus initiating the fusion process (93). DIII is located at the C-terminal end of the protein (Fig 1.5A). It is non-glycosylated and contains a single disulfide bridge which is the critical determinant for proper folding of DIII (84). It is composed of ß-sheets and random coils and adopts an Ig-like fold. DIII is the putative receptor binding domain (6, 13, 17).

The surface structure of flavivirus virions was determined by cryo-EM and image reconstructions using fitting of the crystal structures of soluble E proteins from DEN and WN viruses into the electron density of the virions (51, 66, 110) revealing the structural organization of the E protein on the surface of immature and mature viral particles, respectively (Fig. 1.5C and D).

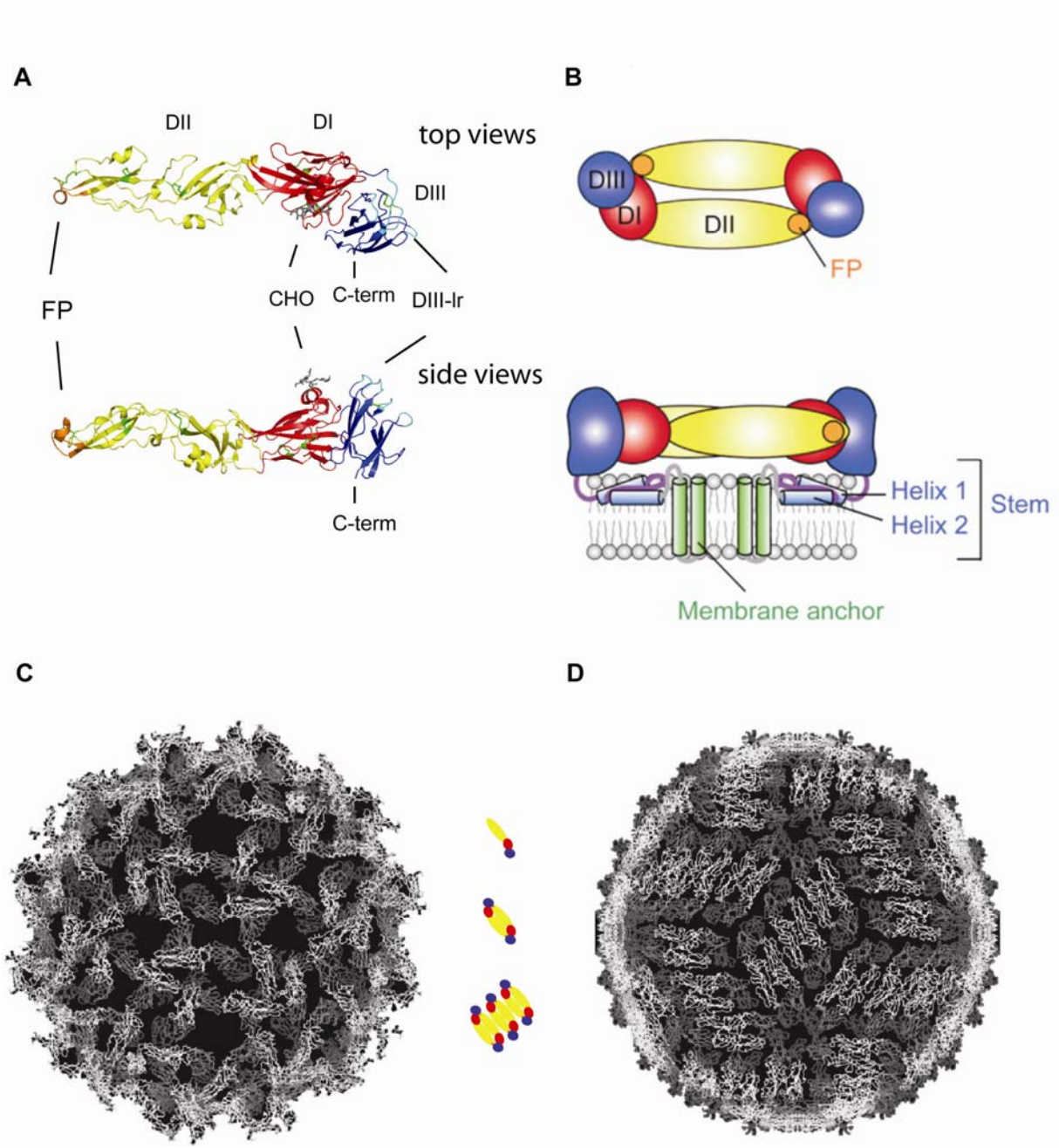

Fig. 1.5 Structural organization of the E protein in its isolated form and on the surface of viral particles.

(A) Ribbon diagram of the WN virus sE monomer, top and side view (68). The lateral ridge of DIII (DIII-lr) is highlighted in cyan and glycosylation (CHO) is indicated in grey.

(B) Schematic representation of the E protein dimer, top and side view (93). The membrane-proximal stem region is shown in blue (Helix 1, 2) and purple (linker) and the transmembrane helices are shown in green. In all panels, DI is indicated in red, DII in yellow and DIII in blue. The fusion peptide (FP) is shown in orange.

(C) Surface structure of immature flaviviruses based on fitting of the crystal structures of soluble E proteins into the cryo-EM electron density of the particle, showing the spiky projections formed by the E-prM heterodimers (60).

(D) Surface structure of mature flaviviruses based on fitting of the crystal structures of soluble E proteins into the cryo-EM electron density of the particle, showing the herring bone-like arrangement of the E protein dimers (60). Figure adapted from (60, 93).
Schematics of an isolated monomer, an E dimer and a raft of three E dimers are depicted between panels C and D.

1.3.4.2 Surface glycoproteins prM/M

The envelope glycoprotein prM is the precursor of the M protein. prM has a molecular mass of about 26 kDa and is anchored in the viral membrane via two alpha helices (Fig. 1.3). During biosynthesis of the structural proteins in the host cell, prM folds independently and is believed to serve as a chaperone for the correct folding of the E protein (62). In immature virions, prM forms heterodimers with the E protein. This interaction shields the fusion peptide at the tip of DII of E (Fig. 1.3 and 1.4) and prevents E from initiating premature fusion of the viral with host cell membranes upon encountering acidic pH in the TGN during the process of secretion (Fig 1.4). In the TGN, prM gets cleaved by the host serine protease furin into the pr and the M protein. Following cleavage and secretion of the viral particle, the pr part falls off upon neutral pH into the extra cellular milieu (57, 109).

1.3.4.3 Capsid protein C

The capsid protein is a highly basic protein with a molecular mass of about 11kDa. Crystal structures have shown that the positively charged residues, which are clustered at the C and N termini, are separated by a hydrophobic stretch and that the basic building blocks of the viral capsid are C protein dimers (22, 49). The molecular architecture of C suggests that the hydrophobic part is involved in interaction with the viral membrane and the positively charged part in interaction with the negatively charged RNA genome (22).

1.4 The antibody response to flaviviruses

Infections with flaviviruses induce antibodies which are specific for structural as well as non-structural proteins. So far, antibodies specific for E, prM/M, C, NS1, NS3 and NS5 have been described (5, 21, 75, 98, 106). Because of its important functions in the flavivirus life cycle, the E protein is the major inducer of virus neutralizing and protective antibodies. Antibodies to prM can also lead to neutralization, presumably through the interaction with partially mature virions (24, 46). NS1-specific antibodies seem to protect via a complement mediated effector mechanism such as reviewed in (21). Using monoclonal antibodies, binding sites of neutralizing antibodies were identified in all three domains of the E protein (4, 15, 30, 38, 69, 70, 82, 83, 87, 96, 98, 107). As reviewed in (82) and (21), DIII contains epitopes of flavivirus type-specific and/or serocomplex-specific antibodies with a high neutralizing potency. Studies with mouse monoclonal antibodies (mabs) suggest that the most potent antibodies bind to the highly surface-exposed upper lateral ridge of DIII (DIII-lr; Fig. 1.5A), as it was shown for WN (69) and DEN viruses (30). The E protein also induces broadly flavivirus cross-reactive antibodies which react even with distantly related flaviviruses from different serocomplexes (Fig. 1.1) but do not, or not completely, neutralize virus infectivity at physiological antibody concentrations (67, 72, 87, 94). It was shown that broadly cross-reactive antibodies predominantly bind to a region containing the fusion peptide (FP), which is located at the tip of DII of the E protein (Fig. 1.3A) (15, 72, 94). The physical explanation for the moderate or lacking neutralizing activity of such antibodies is that the fusion peptide is occluded in mature infectious virions, and therefore not or only poorly accessible for antibody binding (94). As a further evidence, cryo-EM studies of West Nile virus in complex with FP-specific monoclonal antibody E53 indicated that some antibodies seem to bind more efficiently to spikes in immature flaviviruses but not to mature virions, suggesting that the FP-loop is more accessible for antibody binding in the prM-E heterodimer than in the E homodimer (11).

1.4.1 Mechanism of antibody-mediated flavivirus neutralization

The neutralizing potential of an antibody is dependent on the affinity for its target and on the localization and accessibility of the epitope on the viral surface. Neutralization of virus infectivity is achieved when a certain threshold of bound antibodies is exceeded (76). Surface-exposed epitopes like the lateral ridge in DIII (DIII-lr; Fig. 1.5A) require a smaller fractional occupancy than poorly accessible ones, as it is the case with the FP loop in DII (Fig. 1.5A) recognized by broadly cross-reactive antibodies. Neutralisation of infectivity is achieved by preventing functional activities of the E protein, like attachment to the host cell by direct blocking of receptor binding, and/or membrane fusion in the endosome (69, 97, 101). In vivo, additional mechanisms, for instance Fc-receptor and complement-mediated effector functions induced by virus-antibody complexes contribute to protection (75).

1.4.2 Fine-specificity of the polyclonal antibody response to the viral envelope protein E

The E protein is the major surface antigen of flaviviruses and the major target of virus neutralizing and protective antibodies. Analyses of the fine-specificity of the polyclonal antibody responses to important functional determinants of the E protein, like the FP loop and DIII (Fig. 1.5A), in post-infection sera of humans, mice, and horses revealed significant differences among the different species. Mice experimentally infected with West Nile virus showed induction of a high proportion of DIII-specific antibodies, which contributed significantly to WNV neutralization (71). In contrast, no significant proportions of DII FP loop-specific antibodies were detected. Analysis of human and equine DEN and WN virus post-infection sera showed that DIII-specific antibodies are not as dominant as in mice but represent only a minor fraction of the total antibody response (5, 71, 86, 98, 104). In some cases, DIII-specific antibodies contributed significantly to neutralization in humans or horses (16, 86). In contrast to mice, human post-infection sera showed that DII FP-specific antibodies comprise a major fraction in the polyclonal antibody response (16, 53, 71, 98).

Compared to E, antibody responses to the structural proteins prM/M contribute less to virus neutralization. Monoclonal antibodies isolated from mice and humans specific for prM showed poor or no neutralizing activity in vitro and protective efficacy in vivo (5, 24, 44, 46, 98). In infected humans, prM antibodies represent only a minor fraction of the total antibody response (5, 53, 98) to infection with DEN or WN viruses. In the case of dengue virus infections, however, antibodies to prM are believed to contribute to an infection–enhancement phenomenon leading to dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) (19, 44, 81).

1.4.3 Vaccination strategies for the induction of a neutralizing antibody response

Different vaccination strategies to prevent disease caused by flavivirus infection have been pursued in past decades. Until now, vaccines against Japanese (formalin inactivated whole virus and live attenuated), tick-borne encephalitis (formalin inactivated whole virus) and Yellow fever (live attenuated) have been successfully applied in humans (77). Currently, no vaccines against dengue or West Nile viruses are available for humans. For horses, however, WN vaccines (inactivated whole virus, recombinant DNA, live chimeric virus) have been licensed and are currently in use (18).

Alternative approaches which are currently under investigation include recombinant DNA vaccines (78), recombinant subviral particles (37, 61), soluble recombinant antigens (14, 34, 59) and genetically engineered live attenuated viruses. The latter are based on the established YF virus vaccine strain 17D in which the genes coding for prM and E are replaced by the corresponding sequences of heterologous flaviviruses, e.g. JE, DEN or WN viruses (chimeric viruses) (33).

In the context of this thesis, the major interest was in the use of recombinant soluble forms of the E protein (sE) or isolated DIII as immunogens. For immunization of mice (7, 14, 56, 58) monkeys (14, 59, 80) or hamsters (7, 105), recombinant sE proteins from WN and DEN viruses were produced primarily in insect cells. To increase the immunogenicity, sE was administered in combination with adjuvants like Freunds aduvant (14, 56), ISCOMATRIX (105) or aluminium hydroxide (7, 14, 56, 80). Collectively these studies showed that sE induced virus neutralizing antibodies

which were partly or completely protective in vivo as demonstrated by viral challenge of immunized animals.

In the case of recombinant DIII, additional attempts to increase the immunogenicity included not only the application of adjuvants but also the use of heterologous fusion partners (1, 108), expression on viral carriers (48, 92) and strings composed of multiple copies of DIII (10, 47). Administration of high doses of up to 100µg per immunization, the use of strong adjuvants and the administration of at least 2-3 doses were generally required to induce a measurable neutralizing DIII-specific antibody response. Even then, only partial protection from viral challenge could be achieved (1, 12, 40, 89, 108).

Recombinant antigens were not only administered as single antigens but also used in prime-boost vaccination regimens to increase the level and the persistence of neutralizing antibodies. In mice and monkeys, combinations of a DNA vaccine encoding DEN sE, a recombinant DEN DIII fusion protein, with maltose binding protein as a fusion partner, and inactivated whole DEN virus were compared to the single antigens alone (88, 90). However, the chosen prime-boost strategies did not result in an increased neutralizing antibody response compared to homologous immunizations with the DIII protein or the inactivated virus alone. In another study (99) monkeys were primed with recombinant DEN virus DIII and boosted with infectious DEN virus or vice versa. Both regimens did not yield higher antibody titers compared to infection without DIII priming.

1.4.4 Influence of pre-existing antibodies on subsequent responses to infection or immunization

Pre-existing antibodies, acquired either by infection or immunization, can have an impact on the antibody response to subsequently encountered related antigens. In general, inhibition as well as enhancement of the antibody responses have been observed. These phenomena were termed ‘Original Antigenic Sin’ (OAS) and ‘antibody-mediated feedback regulation’.

OAS describes the finding that sequential infections or immunizations with cross-reactive antigens can lead to an accelerated response to the original antigen. In particular, memory responses to epitopes which are shared by the cross-reactive

antigens are primarily activated, whereas the primary response to other epitopes of the subsequently encountered antigen can be impaired or delayed. This phenomenon was originally described for sequential infections with different influenza virus strains in humans (27). OAS phenomena in the context of infections or immunizations with flaviviruses have been described for sequential dengue virus infections with different serotypes (36) or TBE virus vaccination of a YF virus immune individual (43).

Pre-existing serum antibodies can form immune complexes with subsequently encountered antigens and either inhibit or enhance the antibody response by different mechanisms summarized under the term ‘antibody mediated feedback regulation’ (42, 74). Several possibilities were proposed how antigen in complex with immunoglobulin G (IgG) can mediate the suppression of antibody responses, primarily by preventing B-cell activation. First of all, immunoglobulin associated with antigens can mask the respective epitopes by making them inaccessible and therefore prevent the recognition by, and the activation of specific B-cells. Secondly, immune complexes can bind to Fc-gamma receptors (FcyR), for instance on macrophages or dendritic cells, inducing the internalization of the complex thus eliminating the antigen. Finally, cross-linking of the B-cell receptor with inhibitory motifs in the FcyIIB receptor can inhibit the activation of specific B-cells and subsequently, the production of specific antibodies.

Enhancement of antibody responses as a result of the association of immune complexes with activating Fcy receptors on B-cells thus inducing their activation, is a well established mechanism (41).

1.5 Recombinant protein expression

Recombinant proteins can be produced in a variety of different and commercially available expression systems. In general, cell-free and cell-based systems can be distinguished. The cell free systems, in which proteins are synthesized in an in vitro reaction, are expensive and do not achieve the high protein yields of cellular expression systems. Therefore they are of minor relevance and not discussed further. The choice of a suitable expression system with respect to the production of proteins for immunization depends on the specific characteristics of the proteins like

folding properties and post-translational modifications, as well as the required yield and the desired application.

1.5.1 Heterologous protein expression in prokaryotic cells

In general, bacterial expression is suitable for the production of small, self folding proteins without any post-translational modifications. The advantages are the fast growth rates of bacteria and the high expression level of the recombinant protein of up to or even more than 50 % of the total cellular protein. In addition, cultures are relatively easy to handle and production is cheap. A limitation of this approach is that commonly used expression hosts like E.coli do not have the machinery for proper post-translational modifications like glycosylation which can have an impact on the folding and functionality of the protein. In addition, the highly over-expressed recombinant proteins tend to aggregate in the bacterial cell, forming so-called insoluble 'inclusion bodies'. Solubilisation from the insoluble phase and refolding of solubilised material is possible but there is a chance to obtain misfolded protein. Strategies to circumvent this problem are the use of fusion partners which can enhance the solubility (Nus-, GST- or thioredoxin-tags) of the proteins. For flaviviruses, properly folded DIII proteins were produced successfully in bacteria (6, 69, 80, 102, 103).

1.5.2 Heterologous protein expression in eukaryotic cells

Compared to bacteria, high yield expression systems using eukaryotic cells have the advantage that recombinant proteins are properly post-translationally processed (e.g. glycosylation, phosphorylation or the addition of fatty acids) which can impact the folding, stability and thus the functionality of the protein. In addition, inclusion bodies are not formed in the cytosol. However, culturing cells is more expensive and mostly more demanding and expression levels are not as high as it is the case with bacteria. Yeast, insect, and mammalian cells have successfully been applied for the production of recombinant flavivirus proteins.

1.5.2.1 Protein expression in yeast

Using yeast expression systems, recombinant proteins can either be produced as soluble forms, which are secreted into the culture medium and purified, or as transmembrane forms which are displayed on the yeast cell surface. This method, the so-called “yeast surface display”, is a widely used assay format to study protein-protein interactions, including epitope mapping with monoclonal antibodies (mabs) (70, 72, 95, 96). Post-translational modifications like glycosylation can differ from those in higher eukaryotes like insect or mammalian cells. Subviral particles (61), DIII (23) as well as sE proteins (100) were produced in the yeast Pichia Pastoris and obtained in a proper conformation.

1.5.2.2 Protein expression in insect cells

Insect cells are able to perform post-translational modifications of proteins (glycosylation, acylation, disulfide bond formation, proteolytic processing and oligomerisation) similar to mammalian cells, and thus represent suitable hosts for the production of biologically active proteins in its proper conformation. Cells can be grown at a high density in suspension cultures, which can easily be scaled up. The two most widely used expression systems are the Drosophila expression system using Drosophila melanogaster cells and the Baculovirus expression system using Spodoptera frugiperda cells. The Drosophila expression system allows the stable transfection of the host cells for continuous production of recombinant proteins. The baculovirus expression system uses a lytic recombinant virus (Baculovirus) as a vector to deliver the recombinant gene into the host cell. Both systems were used to produce flavivirus proteins for different purposes like structural studies or the production of antigens for ELISA and immunization experiments. As demonstrated by the crystallization of sE proteins from DEN (64, 65) and WN viruses (45, 68) produced in Drosophila and Spodoptera cells, respectively, both systems are suitable to produce sE proteins with a proper conformation. In immunization studies it was shown that recombinant sEs could elicit a strong specific and neutralizing antibody response (7, 14, 56, 58).

1.5.2.3 Protein expression in mammalian cells

Mammalian cells have a highly advanced protein processing machinery and therefore ensure the production of recombinant proteins with proper post-translational modifications, conformation and functionality. For example, complex post-translational modifications like special types of N-linked glycosylation are only possible in mammalian cells and may be necessary especially for the production of recombinant proteins of mammalian origin. Disadvantages are often low yield, relatively slow cell growth and demanding cell culture. The most widely used cell types are CHO, HeLa, Cos-1 or HEK 293T cells. In the case of flaviviruses, recombinant subviral particles (RSPs) have been produced in HeLa (50) and Cos-1 (2, 26, 28) cells, which were also used for the expression of soluble E (37).

2. Objectives

E protein specific antibodies induced by infection or immunization are the major mediators of neutralization and protection against flaviviruses. The determination of the atomic structure of the E protein together with epitope mapping studies using monoclonal antibodies revealed that the E protein induces antibody populations with different functional characteristics, depending on the location of the epitope on the protein. Serocomplex or virus specific antibodies, some of which are known to be the most strongly neutralizing antibodies described so far, map to highly surface-exposed regions of DIII. In contrast, broadly flavivirus cross-reactive antibodies, which are only weakly or non-neutralizing, mainly bind to a region including the fusion peptide (FP) in DII. It is currently unknown whether there is an immunodominance of specific structural entities of E in the course of a polyclonal immune response induced by immunizations with inactivated virus and recombinant subunit antigens of flaviviruses.

The main goal of this thesis was to investigate whether immunodominance and functional quality of the antibody response to E, or individual antigenic regions of E, are influenced by the structural context in which the antigen is presented to the immune system (formalin inactivated whole virus vs. soluble E vs. isolated DIII). It was a further objective to study if the antibody response can artificially be focussed to targets of highly neutralizing antibodies by the application of specifically designed prime-boost immunization regimens. Such strategies could have possible implications for future vaccine design. For those purposes, mice were immunized with different combinations of whole inactivated virus, recombinant soluble sE and isolated DIII of West Nile virus and the contribution of DIII- and FP-specific antibodies to the total neutralizing antibody response was analyzed.

In order to accomplish these goals, the thesis had to be structured into four sub-projects. The first part was dedicated to the production and characterization of recombinant sE and DIII proteins for the immunization of mice and for the use as antigens in immunological assays. Secondly, the conditions to elicit a robust antibody response with the recombinant sE and DIII antigens in mice were determined in a preliminary dose-finding immunization experiment. In order to be able to analyze the fine specificity / immunodominance of the antibody responses in mice, immunological assays such as different ELISA or antibody depletion formats were developed. In the

last part of the thesis, sequential prime-boost immunization regimes were applied in mice using the WN virus antigens and the resulting antibody responses were analyzed with an array of functional and binding assays.

3. Part I: Publication Journal of Virology, in press

Immunodominance and Functional Activities of Antibody Responses to Inactivated West Nile Virus and Recombinant Subunit Vaccines in Mice

Juergen Zlatkovic, Karin Stiasny, Franz X. Heinz*
Department of Virology, Medical University of Vienna, Vienna, A-1095, Austria

published ahead of print on 8 Dec 2010
doi:10.1128/JVI.01886-10

*Corresponding author: Franz X. Heinz, Department of Virology, Medical University of Vienna, Kinderspitalgasse 15, A-1095, Vienna, Austria,
Franz.X.Heinz@meduniwien.ac.at, Tel:+43 1 40490-79500, Fax: +43 1 40490-9795

ABSTRACT

Factors controlling the dominance of antibody responses to specific sites in viruses and/or protein antigens are ill-defined but can be of great importance for the induction of potent immune responses to vaccines. West Nile virus and other related important human-pathogenic flaviviruses display the major target of neutralizing antibodies, the E protein, in an icosahedral shell at the virion surface. Potent neutralizing antibodies were shown to react with the upper surface of domain III (DIII) of this protein. Using the West Nile virus system, we conducted a study on the immunodominance and functional quality of E-specific antibody responses after immunization of mice with soluble protein E (sE) and isolated DIII in comparison to inactivated whole virions. With both virion and sE, the neutralizing response was dominated by DIII-specific antibodies but the functionality of these antibodies was almost 4 times higher after virion immunization. Antibodies induced by the isolated DIII had an at least 15-fold lower specific neutralizing activity compared to the virion and only 50% of these antibodies were able to bind to virus particles. Our results suggest that immunization with the tightly packed E in virions focuses the DIII antibody response to the externally exposed sites of this domain which are the primary targets for virus neutralization, different to sE and isolated DIII which also display protein surfaces that are cryptic in the virion. Despite its low potency for priming, DIII was an excellent boosting antigen, suggesting novel vaccination strategies that strengthen and focus the antibody response to critical neutralizing sites in DIII.

INTRODUCTION

The availability of high resolution structures is a pre-requisite for understanding structural determinants of immunogenicity and immunodominance. Knowledge of factors that control and/or influence these properties of antigens can lead to an improvement of existing vaccines and the rational design of new vaccine antigens and regimens (13). Flaviviruses are among those human-pathogenic viruses for which detailed structural information is available through the combined use of X-ray crystallography and cryo-electron microscopy (cryo-EM) (25, 30, 36-38, 40, 49, 65). The most important representatives are the mosquito-borne yellow fever (YF), dengue (DEN), Japanese encephalitis (JE), and West Nile (WN) viruses, as well as tick-borne encephalitis (TBE) virus (14). These viruses have a significant impact on public health and the potential for emergence in new geographic regions, as exemplified by the expansion of WN virus in the Americas since its first introduction to the USA in 1999 (17). Human vaccines are in general use against YF (live attenuated), JE (live attenuated and inactivated whole virus), and TBE (inactivated whole virus) but not yet against DEN and WN viruses (48).

Mature flavi-virions are composed of an isometric capsid containing the positive-stranded RNA genome and a lipid envelope with two membrane-associated proteins, E and M (33). As revealed by cryo-EM, 90 copies of E protein dimers (oriented parallel to the viral membrane) form a tight shell at the virion surface in a herringbone-like arrangement (30, 38) (Fig. 1A). Because of its dual function in receptor binding and acid pH-induced membrane fusion (33), E is the major target of virus-neutralizing antibodies that mediate protection and long-lived immunity, both after natural infection and vaccination (12, 46, 47, 50). Each of the monomeric subunits of E contains three distinct domains, designated DI, DII, and DIII (Fig. 1B). The central DI is flanked by DII, carrying the highly conserved internal fusion peptide (FP) loop, and by DIII, which has an immunoglobulin-like fold. DIII protrudes slightly off the relatively smooth surface of mature virions and has been implicated in receptor-binding (6, 9, 32). The rest of the molecule, which is absent from the crystal structures, comprises the so-called stem that follows DIII and leads to the double membrane-spanning anchor at the C-terminus of the molecule.

Epitopes involved in virus neutralization have been identified in each of the three domains, at sites exposed at the virion surface (3, 10, 16, 20, 29, 41, 44, 50, 51, 58,

59, 63). Studies with mouse monoclonal antibodies suggest that the most potent neutralizing antibodies are virus type-specific and bind to the highly variable upper lateral ridge of DIII (16, 42). Antibodies to the conserved FP loop, on the other hand, are broadly flavivirus cross-reactive but display comparatively low neutralizing activity (44, 57).

A significant degree of heterogeneity in the specificities of antibody populations was observed with mouse, human, and horse post-infection and/or post-immunization sera (11, 31, 43, 52, 59, 62). Antibodies to DIII made up a significant fraction of the IgG response in mice, but only a small proportion in humans in whom the response was dominated by antibodies to the FP loop (11, 31, 43, 59). Factors that control the immundominance of antibody responses are ill-defined but, in addition to host-specific factors, may be influenced by the physical organization of the antigen and differences in the presentation of antigenic protein surfaces during immunization. The specific design and selection of antigens can also provide clues to the development of vaccines against highly variable pathogens, such as HIV or influenza viruses (15, 55, 61).

Considering the many unresolved questions of immunodominance, we conducted a mouse immunization study - using the WN virus system - in which we investigated whether and in which way the fine-specificity of antibody responses to E, especially the contribution of DIII antibodies, was influenced by the quaternary organization of the immunogen, i.e. E in its isolated monomeric form (sE) as compared to E tightly packed in whole inactivated virus. We also assessed whether it is possible to specifically focus and strengthen the antibody response to DIII by i. boosting with isolated DIII after priming with inactivated virion or sE and ii. priming with isolated DIII followed by boosting with inactivated virion or sE. We demonstrate that inactivated virions induced DIII-reactive antibodies with higher specific neutralizing activity than sE and that in both cases antibodies to this domain dominated the total neutralizing activity. We also show that the isolated DIII is an excellent booster antigen which restimulates DIII-specific and neutralizing antibodies after priming with virion or sE more strongly than the homologous priming antigens. Paradoxically, priming with DIII followed by boosting with virion or sE had no beneficial effect but – in contrast - resulted in dampening of the antibody response. Our data suggest that the neutralizing immune response to flavivirus vaccines may be focused and optimized,

after appropriate priming, by booster vaccinations with preparations of recombinant DIII.

MATERIALS AND METHODS

Production of purified inactivated WN virus. Virus production was carried out essentially as described in (21) for tick-borne encephalitis virus. In brief, primary chicken embryo cells were infected with WN virus, strain NY99 (GenBank# AF196835). 24 hours p.i. the cell supernatant was harvested, clarified by centrifugation and inactivated with formalin (1:2000) for 24 hrs at 37°C. The inactivated virus was concentrated by ultracentrifugation and purified by rate zonal followed by equilibrium sucrose density gradient centrifugation. SDS-PAGE analysis indicated the presence of a small proportion of uncleaved prM, as is characteristic for purified preparations of WNV (8) and for flaviviruses in general (24) as well as oligomeric bands of C and E as a result of formalin cross-linking (Fig. 1D).

Expression and purification of WN and TBE virus sE. Recombinant E proteins from WN virus strain NY99 and TBE virus strain Neudoerfl (GenBank# U27495) were expressed in Schneider 2 (S2) cells using the pMTBip/V5-His vector (Invitrogen) and inserts containing the coding sequences for prM and E (C-terminally truncated after amino acid 400). For immunization, the WN virus sE was produced without His-tag by the introduction of a stop codon after amino acid 400. Blasticidin-resistance was used for the selection of stably transfected cells according to the manufacturer's instructions. Expression was induced by $CuSO_4$ and the supernatants were harvested 7 to 11 days post-induction. Recombinant proteins were purified by immuno-affinity chromatography using the flavivirus cross-reactive monoclonal antibody 4G2 (ATCC# HB-112). The purity of the recombinant sE proteins was >95%, as determined by SDS-PAGE and densitometric analysis (Fig. 1D).

Expression and purification of WN virus recombinant DIII. DIII of WN virus strain NY99, encompassing amino acids 300 to 399 of E, was expressed with and without a C-terminal His-tag (including a stop codon after amino acid 399) in *Escherichia coli* strain BL21 using the pET 32a Xa/LIC vector (Novagen) as a fusion protein with thioredoxin. For purification of soluble DIII without a C-terminal His tag, the fusion protein in clarified cell lysates was bound to a Ni++-immobilized affinity column (GE Healthcare Life Sciences) via the internal His-tag located between thioredoxin and DIII, and DIII was eluted from the column after proteolytic cleavage

from thioredoxin by factor Xa protease. For expressing C-terminally His-tagged DIII, the internal His-tag was deleted from the expression cassette so that the fusion protein bound to the affinity column via the C-terminal His tag of DIII. The thioredoxin fusion partner was cleaved off by factor Xa, removed by washing the column, and DIII was specifically eluted by an imidazole gradient. Final purification was accomplished by anion exchange chromatography using Sepharose Q columns (GE Healthcare Life Sciences) according to the manufacturer's instructions. By SDS gel analysis, a single band corresponding to DIII was visible by Coomassie staining (Fig. 1D).

Mouse immunization experiments. Mouse experiments were carried out in strict accordance with the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA) and the Austrian federal law. The protocol was approved by the ethics committee of the Medical University of Vienna and the Austrian Federal Ministry of Science and Research (Permit Number: BMWF-66.009/0068-II/10b/2008). Six to eight weeks old C57BL/6 mice (Charles River Laboratories) were immunized sub-cutaneously with inactivated virus, sE or DIII, adsorbed to 0.2% $Al(OH)_3$ (Aluminum hydroxide gel, Sigma) in 100µl buffer containing 0.05M triethanloamine, 0.1M NaCl, pH 8.0, at doses of 1µg, 5µg and 20µg per immunization, respectively. Blood was taken from the tail vein at the time points indicated in Fig. 1E and F, using microvette 200 capillaries (Sarstedt). Antibody analyses were carried out using pools of sera from the groups of mice with the same immunization schedule.

Virion ELISA. Microtiter plates were coated with formalin-inactivated purified WN virus at a concentration of 0.35 µg/ml in carbonate buffer, pH 9.6, at 4°C. Serial three-fold dilutions of pooled mouse sera, starting at a dilution of 1:100, were added to the plates and incubated for 1h at 37°C. As negative controls, sera from naïve mice were included. The bound antibodies were then detected using peroxidase-labeled rabbit anti-mouse immunoglobulin G as described in (57). Titration curves were established using Graph pad Prism 5 software and serum titers were defined at an absorbance (490 nm) cut-off of 0.35, as deduced from the analysis of negative control sera. Each serum pool was tested in at least three independent experiments, and results are presented as the geometric means of these repetitive experiments.

Analysis of avidity in virion ELISA. Samples were analyzed in the standard virion ELISA, except that an additional washing step with and without 6M urea in the washing buffer was applied after serum incubation, essentially as described by (56). Titers were determined at an absorbance cut-off of 1.0 after curve-fitting using a four-parameter logistic regression (GraphPad Prism 5.0). Relative avidities are expressed as percentage of the titer obtained in the presence of urea compared to the titer in the absence of urea.

sE and DIII ELISA. Microtiter plates were coated with rabbit anti-His-tag IgG antibody (QED Bioscience), at 4°C in carbonate buffer, pH 9.6. DIII-His or sE-His proteins were added at a concentration of 1 and 0.5 µg/ml, respectively, and incubated for 1h at 37°C. Three-fold serial dilutions (starting at 1:100) of pooled mouse sera were then added for 1h at 37°C. As negative controls, sera from naïve mice were included. The bound antibodies were detected using peroxidase-labeled goat anti-mouse immunoglobulin G (Pierce) for 1h at 37°C. Visualization of bound antibodies and determination of serum titers were done as described for the virion ELISA.

WN virus neutralization assay. WN virus neutralization assays were carried out in microtiter plates using Vero cells (ECACC #84113001). Pooled mouse sera were heat-inactivated at 56°C for 30 minutes and two-fold serial dilutions, starting at a dilution of 1:20, were incubated with 20-40 $TCID_{50}$ of WN virus, strain NY99, for 1h at 37°C. The virus antibody mixture was then transferred onto Vero cells, and incubated for 4 days at 37°C. The cells were fixed with 4% paraformaldehyde for 30 minutes at room temperature. After fixation, the cells were blocked and permeabilized with Tris-buffered saline (TBS) (50 mM Tris; 150 mM NaCl, pH 7.6, containing 3% Non-fat dry milk, 0.5% Triton X-100 and 0.05% Tween-20) for 30 minutes at 37°C, and a WN virus specific rabbit serum was added for 1.5h at 37°C. Bound antibodies were detected with alkaline phosphatase labeled anti rabbit IgG (Sigma) and SigmaFast pNNp (Sigma) as a substrate. The enzymatic reaction was stopped with 1.5N NaOH and absorbance was measured at 405 nm. Titers were determined after curve-fitting using a four-parameter logistic regression (GraphPad Prism 5.0) and a cut-off of 90% reduction of the absorbance in the absence of antibody (NT_{90}). A cut-off of 50% (NT_{50}) was not as robust in the lower titer range, since some negative sera would

have yielded false positive titers between 10 and 20. Each serum pool was tested in at least two independent experiments.

Antibody depletion using WN virus DIII and TBE virus sE. One µg of His-tagged WN virus DIII or TBE virus sE was incubated with 1mg paramagnetic 'Dynabeads His-Tag Isolation & Pull Down' beads (Invitrogen) for 30 minutes at room temperature on an orbital shaker (1,000 rpm). After pelleting by magnetic force, the beads were resuspended in pull-down buffer according to the manufacturer's instructions and incubated for 1h at 37°C with a 1:5 dilution of serum pools in PBS. The beads were pelleted again by magnetic force and the depleted serum was collected. To achieve quantitative depletion, this procedure was performed three times. Lack of non-specific binding of antibodies to the beads was confirmed by the incubation of mouse immune sera with unloaded beads and beads loaded with an unrelated His-tagged protein.

Antibody depletion using WN virus. 10 µg of inactivated WN virus were incubated with pooled sera (diluted 1:50 in PBS pH 7.4, 0.1% BSA) for 1h at 37°C. The virus antibody complexes were pelleted by ultracentrifugation in a Beckman Ti90 rotor at 50,000 rpm at 4°C for 1h. The supernatant was collected and used for further analyses.

Statistical analyses. Data were analyzed with GraphPad Prism software, version 5 (GraphPad Software Inc., San Diego). Two tailed t-tests were used to compare antibody titers, ratios of antibody titers and ELISA avidities. Differences were considered significant when the p value was less than 0.05.

RESULTS

Characteristics of immunogens. Three different antigens of WN virus were used in our immunization studies (Fig. 1A-D) (see also Materials and Methods): 1. Highly purified formalin-inactivated virus. As revealed by cryo-EM studies with tick borne encephalitis virus (PhD thesis, S.T. Brady, University of Oxford, unpublished data), formalin inactivation does not affect the specific herring bone-like arrangement of 90 E protein dimers at the surface of mature virions. 2. Purified soluble recombinant E (sE) produced in S2 cells, shown to be a monomer by cross-linking and sedimentation analyses. Evidence for correct folding was obtained by the demonstration of the presence of S-S bridges by SDS-PAGE under reducing and non-reducing conditions (data not shown) 3. Purified recombinant DIII produced in bacteria. The presence of the single disulfide bridge was confirmed by mass spectrometry (data not shown).

All three antigen preparations displayed similarly strong reactivities with the conformation-sensitive monoclonal antibodies (mabs) E16 and E24 (kindly provided by Michael S. Diamond; (42)) in the virion as well as the sE and DIII ELISAs as described in Materials and Methods (data not shown).

Consistent with previous data (19), preliminary experiments had revealed that the inactivated virus had an at least 6-fold higher specific immunogenicity per µg of antigen than sE (data not shown). DIII alone did not yield significant titers even when used at a dose of 50µg. Therefore – in order to obtain measurable antibody responses and to be able to assess modulations in fine-specificity and immunodominance – $Al(OH)_3$ was used as an adjuvant and different antigen doses per mouse were applied at each immunization as follows: inactivated virion: 1µg; sE: 5µg; DIII: 20µg.

Antibody response to virion, sE and DIII. For investigating the influence of the immunogens' structural context (i.e. the same antigen tightly packed in virions or in soluble form) on fine specificity, dominance and functional activity of the antibody response, we immunized groups of mice twice with whole inactivated virus, the isolated sE and the isolated DIII (see immunization schedule in Fig. 1E). Eight weeks after the second immunization, blood samples were taken and pooled sera were analyzed in neutralization assays as well as in ELISAs using inactivated virus, sE,

DIII and the heterologous TBE virus sE as antigens (Fig. 2). Both, the inactivated virion and sE, induced neutralizing antibodies, but the titer was higher after virion immunization - despite the fact that the dose applied was only one fifth of that of sE (Fig. 2A). DIII, on the other hand, induced antibodies that were only partially neutralizing (NT_{50} titer of 35), and the extrapolation of the neutralization curve indicated that the NT_{90} titer was below 10. In ELISAs, the sera exhibited strikingly different patterns of reactivities with virion, sE, DIII and TBE virus sE (Fig. 2B), suggestive of differences in the fine-specificities of the antibody responses obtained. Most importantly, the E protein in the context of whole virions induced a lower proportion of DIII antibodies – relative to the total amount of virion reactive antibodies – than the isolated sE (Fig. 2C).

Contribution of DIII-specific antibodies to virus neutralization. Because of the different ELISA ratios obtained after immunization with virion and sE (Fig. 2B and C) and the fact that the most potent neutralizing mabs described so far are directed to DIII (12, 46), we investigated the contribution of DIII-specific antibodies to neutralization by their removal with recombinant DIII bound to magnetic beads (Materials and Methods). As shown by DIII-ELISA, this procedure resulted in a virtually complete depletion of DIII-reactive antibodies from the serum pools (Fig. 3A); in virion ELISA, the drop of reactivity was 32% with the virion sera but as high as 76% with sE sera (Fig. 3B). In both instances, however, about 75% of the neutralizing activity was removed and thus attributable to DIII-reactive antibodies (Fig. 3C). This discrepancy reflects significant differences in the specific neutralizing activities of DIII-reactive antibodies induced by virion and sE, expressed as ratios of DIII-associated NT vs. DIII ELISA x 100 (Fig. 3D). These values were 1.5 and 0.4 after immunization with virion, and sE, respectively, and <0.1 after DIII immunization (no measureable NT_{90} titer). One possible explanation of these findings would be that E in its soluble form, and even more so the isolated DIII, would induce antibodies also to sites that are not highly exposed at the virion surface and thus contribute less to virus neutralization (46).

To corroborate this hypothesis and to identify such antibodies, we depleted the three serum pools with whole inactivated virus and analyzed the resulting sera in virion and DIII ELISAs (Fig. 4A and B). With all three samples, the virus-reactive antibodies were almost completely removed by the procedure used (Fig. 4A). The

DIII ELISA revealed that about 50 % of the antibodies induced by DIII did not react with the virus in solution (Fig. 4B). Unexpectedly however, no difference was found between the virus- and sE-induced DIII-specific antibodies in this assay (Fig. 4B). A possible additional explanation of the lower functional activity of DIII-specific antibodies induced by sE and DIII compared to the virion could be related to differences in avidity for the virus. Such differences would be obscured in the depletion procedure because of the high excess of virus. We therefore analyzed the relative avidities of the sera in virion ELISAs using a urea wash step (Materials and Methods). As displayed in Fig.5, significant differences were found in the expected order, with the highest avidity for the antibodies induced by virus, followed by sE and DIII.

Induction of broadly cross-reactive antibodies. Broadly cross-reactive antibodies directed to the highly conserved FP loop can make up a significant proportion of the polyclonal immune response to flaviviruses (11, 31, 43). For determining such antibodies, we analyzed the post-immunization sera in an ELISA with the heterologous TBE virus sE as an antigen. Overall, the WN virion and WNV sE post-immunization sera displayed only very low cross-reactive titers in this assay (Fig. 2B) and depletion with TBE virus sE did not measurably diminish their reactivities with WN virus sE (data not shown). There was also no evidence for cross-neutralization in assays with TBE virus (data not shown). As expected, due to the lack of the conserved FP site, WNV DIII did not induce any detectable cross-reactive antibodies (Fig. 2B), consistent with previous studies on the antigenic specificity of DIII (4).

Modulation of immunodominance by prime-boost regimens with DIII. In order to increase and strengthen the antibody response towards DIII and concomitantly the neutralizing potency of post-immunization sera, we conducted two sets of experiments: 1. mice primed with either virion or sE were boosted with DIII, and 2. mice were primed with DIII and boosted with virion or sE (compare immunization schedule in Fig. 1F). The resulting sera were pooled and analyzed in virion- and DIII-ELISAs as well as in virus neutralization assays. As shown in Fig. 6, DIII proved to be an excellent booster antigen, despite its low immunogenicity when used as a single immunogen (see Fig. 2A, B). In combination with inactivated virus

as well as with sE, it strongly boosted DIII- and virus-reactive antibodies in ELISA (Fig. 6A, B, D, E) which was associated with a strong increase of neutralizing activity (Fig. 6C, F). This effect was more pronounced with virion- than with sE-primed mice (Fig. 6A-C and D-F, respectively) and resulted in significantly higher neutralization titers as compared to the controls which received the inactivated virus for priming as well as for boosting (Fig. 6C).

The alternative schedule, i.e. priming with DIII and boosting with virion or sE had a quite unexpected outcome. In both instances, pre-immunization with DIII resulted in an impairment of the antibody response - measured in ELISA and neutralization – as compared to unprimed controls (Fig. 7).

DISCUSSION

When designing novel vaccines, it would be desirable to use immunogens and immunization schedules with an optimized immunological performance by focusing the response to those sites that are expected to induce the most potent neutralizing antibodies. This may be achieved by the selection or specific design of the immunogen (15, 55, 61) and/or by designing specific prime-boost regimens that direct and strengthen the immune response to most desirable functional parts of the antigen. In the case of flaviviruses, there is evidence (mostly from the analysis of mouse monoclonal antibodies (16, 42, 59)) that DIII of the envelope protein E can induce potent neutralizing antibodies which are directed to the upper lateral ridge of this domain (12, 47).

In our study, we demonstrate that the functional quality of polyclonal DIII-specific antibody responses was strongly dependent on the structural context of this domain in the immunogen. The differences found were substantial. DIII-specific antibodies induced by the inactivated virus had an almost four-fold higher neutralizing activity (relative to the DIII ELISA titer; Fig. 3D) than those induced by soluble E. The discrepancy between ELISA-reactivity and functional activity was even more pronounced with antibodies induced by the isolated DIII which had a more than 15-fold lower specific neutralizing activity than those obtained after virion immunization and failed to completely neutralize the virus. These differences were also reflected by the overall avidities of the antibodies to the virion which were shown to be highest after immunization with inactivated virus, intermediate with sE, and lowest with DIII. These findings are in agreement with a dengue type 2 immunization study in rhesus macaques (53) using inactivated virion, a DNA vaccine expressing prM and E, and a recombinant DIII-maltose-binding fusion protein. Also in this case, the antibodies induced by the inactivated virus had the highest avidity towards the virus and this property correlated with a reduction in the total days of viremia after challenge (53).

It is likely that the phenomena observed in our study are related to differences in the exposure of antigenic sites in DIII and sE that are buried or less accessible in the virus. Antibodies to such sites may not reach an occupancy at the virion surface that is required for efficient neutralization (46). Inspection of the virion structure shows that DIII forms the highest protrusion in the mature virus, but that only a fraction of the total surface of DIII is accessible to the binding of antibodies. Substantial parts of

potentially antigenic sites are shielded in the context of the mature virion, specifically those at the bottom of the domain – directed towards the viral membrane – and those at its lateral sides because of interactions between E dimers in the tightly packed icosahedral envelope of the virus (Fig. 1A). The location of DIII epitopes as a decisive factor in virus neutralization is best exemplified by the WN virus-specific E16 mab that has been characterized by X-ray crystallography as well as cryo-EM (26, 41) and the mabs 5A2-7, 13D4-1 and E111 (raised against dengue virus) that have been mapped by yeast display combined with mutagenesis (58). E16 and related mabs are strongly neutralizing and bind to sites at the upper lateral ridge of DIII (Fig. 1C) that are highly accessible in the context of whole virions (16, 26, 41, 42). In contrast, the epitopes defined by mabs 5A2-7, 13D4-1 and E111 include the conserved AB loop at the bottom of DIII. This site is exposed in isolated or yeast-displayed DIII but has limited exposure in the context of the virus, because it is located inside the shell of E protein dimers and faces the viral membrane of the mature virion (58). The corresponding mabs therefore are poorly or not neutralizing. It has to be kept in mind however, that the E proteins in flaviviruses can undergo dynamic movements at the virion surface at 37°C ('breathing'), thus allowing virus neutralization also by antibodies to partially occluded sites (34). Furthermore, the accessibility of antigenic sites may also be influenced by the presence of partially immature virions in the virus neutralization assay (39). Whether and to which extent such phenomena can also have an influence on the dominance of antibody responses to certain antigenic sites remains to be elucidated.

The relative dominance of DIII-specific antibodies in our study (32% and even 76% of the total antibody response after immunization with virion and sE, respectively) is consistent with other reports on antibody responses to flavivirus infections and immunizations in mice (43). In contrast, several studies with flavivirus-infected humans (11, 31, 43, 59, 62) and horses (52) do not provide evidence for a dominance of DIII antibodies but suggest that the antibody response is skewed to potentially less potent epitopes in DI and DII, including the broadly cross-reactive fusion loop (11, 31, 43, 59).

In the monomeric WN virus sE, the FP loop is highly exposed at the tip of DII (Fig. 1B) (25, 40). It was therefore surprising that – like with whole virions – only low titers of broadly cross-reactive antibodies were induced by this antigen. In both instances, this response was minimal relative to the total anti-virion response. Given the strong

dominance of FP loop-specific antibodies reported in studies using human post-infection sera (11, 31, 43, 59), this is an additional indication for significant differences between mice and humans in the immunodominance of individual epitopes. The factors responsible for directing the antibody response to selected sites at the surface of the same antigen in different species are unknown and will have to be elucidated for generating a more rational basis of engineering antigens for future vaccine design.

Consistent with published data, the immunizing potency of DIII alone was low in our study and different attempts have been described in the literature to increase its immunogenicity (reviewed by (18)). This includes the use of fusion proteins (1, 22, 35, 64), combinations with strong adjuvants such as Freund's adjuvant (1, 2, 22, 35), the use of strings of DIII (7, 27), expression by adenovirus vectors (28), or the coupling to large carriers such as a bacteriophage (54) to mimic its presentation at the surface of the virus. However, despite its low immunogenicity when used alone, DIII proved to be an excellent boosting antigen and strongly enhanced the neutralizing antibody response of mice pre-immunized with inactivated virion or sE. In this respect, DIII was superior to the more complex immunogens used for priming. It is justified to assume that DIII (in the prime-boost regimen applied) primarily induced an anamnestic response to those sites of DIII presented at the virion surface that are relevant for virus neutralization. Considering the lack of immunodominance of DIII in humans, novel vaccination strategies could be based on the exploitation of the superior specific immunogenicity and priming capacity of inactivated virions combined with the excellent boosting capacity of DIII. Such an immunization regimen could foster the antibody response to the most relevant neutralizing site in the virus and thus potentially lead to a strengthening of the immunity induced by vaccination. Similar boosting approaches would also be possible in combination with live flavivirus vaccines (60).

As an alternative for focusing the antibody response towards DIII, we also investigated a regimen of priming with recombinant DIII followed by boosting with the more complex antigens, i.e. virion and sE. Surprisingly, this schedule resulted in a significant negative effect on the total antibody response against the booster antigens and, most importantly, in an apparent impairment of the neutralizing response. Factors proposed to be involved in such negative effects include T cell mediated suppression, masking pre-existing antibody, or enhanced clearance of immune

complexes (13, 23, 45). The phenomenon observed may also be influenced by the specific combinations of doses and the immunization schedule used in our study, since opposite effects i.e. enhanced neutralizing antibody responses after priming with recombinant DIII followed by live virus infection were described in a dengue virus study (5). Possible effects of using different combinations of antigens, doses and live virus immunizations on the immunodominance of specific sites may be a topic of future investigations. In any case, our data point out that the use of the isolated DIII for priming can lead (at least under certain circumstances) to an unwanted negative effect and some caution should therefore be applied in such immunization regimens.

Overall, our study emphasizes that the functionality of antibodies induced by WN virus antigens is not only dependent on their native conformation but also strongly influenced by their quaternary organization. The flavivirus particle displays the sites most relevant for virus neutralization at its surface, whereas other potentially antigenic faces of the envelope proteins are occluded due to their tight packing in an icosahedral lattice. Such sites, however, are exposed in isolated recombinant proteins which are therefore potentially prone to induce a higher proportion of antibodies that are irrelevant for neutralization. Despite its low specific immunogenicity when used alone, DIII could be superior as a booster vaccine compared to virion or sE, because it optimizes the development of neutralizing antibodies and leads to an increase of the immunodominance of the most critical neutralizing determinants in the virion.

ACKNOWLEDGMENTS

This work was supported by intramural funds of the Medical University of Vienna.

We gratefully acknowledge the technical assistance of Oksana Vratskikh and Karen Pangerl in conducting the mouse experiments, Walter Holzer for virus production and Jutta Hutecek for neutralization assays. We thank Michael S. Diamond for providing hybridomas secreting E16 and E24 monoclonal antibodies and Tim Skern for critically reading the manuscript.

Potential conflict of interest: Franz X. Heinz is a member of the Scientific Advisory Board of Intercell, an inventor of patents on flavivirus vaccines, and has consulted Baxter for developing tick-borne encephalitis vaccines.

REFERENCES

1. **Alka, K. Bharati, Y. P. Malik, and S. Vrati.** 2007. Immunogenicity and protective efficacy of the E. coli-expressed domain III of Japanese encephalitis virus envelope protein in mice. Med Microbiol Immunol **196:**227-31.
2. **Babu, J. P., P. Pattnaik, N. Gupta, A. Shrivastava, M. Khan, and P. V. Rao.** 2008. Immunogenicity of a recombinant envelope domain III protein of dengue virus type-4 with various adjuvants in mice. Vaccine **26:**4655-63.
3. **Beasley, D. W., and A. D. Barrett.** 2002. Identification of neutralizing epitopes within structural domain III of the West Nile virus envelope protein. J Virol **76:**13097-100.
4. **Beasley, D. W., M. R. Holbrook, A. P. Travassos Da Rosa, L. Coffey, A. S. Carrara, K. Phillippi-Falkenstein, R. P. Bohm, Jr., M. S. Ratterree, K. M. Lillibridge, G. V. Ludwig, J. Estrada-Franco, S. C. Weaver, R. B. Tesh, R. E. Shope, and A. D. Barrett.** 2004. Use of a recombinant envelope protein subunit antigen for specific serological diagnosis of West Nile virus infection. J Clin Microbiol **42:**2759-65.
5. **Bernardo, L., L. Hermida, J. Martin, M. Alvarez, I. Prado, C. Lopez, R. Martinez, R. Rodriguez-Roche, A. Zulueta, L. Lazo, D. Rosario, G. Guillen, and M. G. Guzman.** 2008. Anamnestic antibody response after viral challenge in monkeys immunized with dengue 2 recombinant fusion proteins. Arch Virol **153:**849-54.
6. **Bhardwaj, S., M. Holbrook, R. E. Shope, A. D. Barrett, and S. J. Watowich.** 2001. Biophysical characterization and vector-specific antagonist activity of domain III of the tick-borne flavivirus envelope protein. J Virol **75:**4002-7.
7. **Chen, S., M. Yu, T. Jiang, Y. Deng, C. Qin, and E. Qin.** 2007. Induction of tetravalent protective immunity against four dengue serotypes by the tandem domain III of the envelope protein. DNA Cell Biol **26:**361-7.
8. **Cherrier, M. V., B. Kaufmann, G. E. Nybakken, S. M. Lok, J. T. Warren, B. R. Chen, C. A. Nelson, V. A. Kostyuchenko, H. A. Holdaway, P. R. Chipman, R. J. Kuhn, M. S. Diamond, M. G. Rossmann, and D. H. Fremont.** 2009. Structural basis for the preferential recognition of immature flaviviruses by a fusion-loop antibody. Embo J **28:**3269-76.

9. **Chu, J. J., R. Rajamanonmani, J. Li, R. Bhuvanakantham, J. Lescar, and M. L. Ng.** 2005. Inhibition of West Nile virus entry by using a recombinant domain III from the envelope glycoprotein. J Gen Virol **86:**405-12.
10. **Crill, W. D., and G. J. Chang.** 2004. Localization and characterization of flavivirus envelope glycoprotein cross-reactive epitopes. J Virol **78:**13975-86.
11. **Crill, W. D., H. R. Hughes, M. J. Delorey, and G. J. Chang.** 2009. Humoral immune responses of dengue fever patients using epitope-specific serotype-2 virus-like particle antigens. PLoS One **4:**e4991.
12. **Diamond, M. S., T. C. Pierson, and D. H. Fremont.** 2008. The structural immunology of antibody protection against West Nile virus. Immunol Rev **225:**212-25.
13. **Dormitzer, P. R., J. B. Ulmer, and R. Rappuoli.** 2008. Structure-based antigen design: a strategy for next generation vaccines. Trends Biotechnol **26:**659-67.
14. **Fauquet, C. M., M. A. Mayo, J. Maniloff, U. Desselberger, and L. A. Ball.** 2005. Virus Taxonomy. (San Diego: Elsevier Inc.).
15. **Forsell, M. N., W. R. Schief, and R. T. Wyatt.** 2009. Immunogenicity of HIV-1 envelope glycoprotein oligomers. Curr Opin HIV AIDS **4:**380-7.
16. **Gromowski, G. D., N. D. Barrett, and A. D. Barrett.** 2008. Characterization of dengue virus complex-specific neutralizing epitopes on envelope protein domain III of dengue 2 virus. J Virol **82:**8828-37.
17. **Gubler, D. J.** 2007. The continuing spread of West Nile virus in the western hemisphere. Clin Infect Dis **45:**1039-46.
18. **Guzman, M. G., L. Hermida, L. Bernardo, R. Ramirez, and G. Guillen.** 2010. Domain III of the envelope protein as a dengue vaccine target. Expert Rev Vaccines **9:**137-47.
19. **Heinz, F. X., S. L. Allison, K. Stiasny, J. Schalich, H. Holzmann, C. W. Mandl, and C. Kunz.** 1995. Recombinant and virion-derived soluble and particulate immunogens for vaccination against tick-borne encephalitis. Vaccine **13:**1636-42.
20. **Heinz, F. X., R. Berger, W. Tuma, and C. Kunz.** 1983. A topological and functional model of epitopes on the structural glycoprotein of tick-borne encephalitis virus defined by monoclonal antibodies. Virology **126:**525-37.

21. **Heinz, F. X., and C. Kunz.** 1981. Homogeneity of the structural glycoprotein from European isolates of tick-borne encephalitis virus: comparison with other flaviviruses. J Gen Virol **57:**263-74.
22. **Hermida, L., R. Rodriguez, L. Lazo, R. Silva, A. Zulueta, G. Chinea, C. Lopez, M. G. Guzman, and G. Guillen.** 2004. A dengue-2 Envelope fragment inserted within the structure of the P64k meningococcal protein carrier enables a functional immune response against the virus in mice. J Virol Methods **115:**41-9.
23. **Hjelm, F., F. Carlsson, A. Getahun, and B. Heyman.** 2006. Antibody-mediated regulation of the immune response. Scand J Immunol **64:**177-84.
24. **Junjhon, J., T. J. Edwards, U. Utaipat, V. D. Bowman, H. A. Holdaway, W. Zhang, P. Keelapang, C. Puttikhunt, R. Perera, P. R. Chipman, W. Kasinrerk, P. Malasit, R. J. Kuhn, and N. Sittisombut.** Influence of pr-M cleavage on the heterogeneity of extracellular dengue virus particles. J Virol **84:**8353-8.
25. **Kanai, R., K. Kar, K. Anthony, L. H. Gould, M. Ledizet, E. Fikrig, W. A. Marasco, R. A. Koski, and Y. Modis.** 2006. Crystal structure of west nile virus envelope glycoprotein reveals viral surface epitopes. J Virol **80:**11000-8.
26. **Kaufmann, B., G. E. Nybakken, P. R. Chipman, W. Zhang, M. S. Diamond, D. H. Fremont, R. J. Kuhn, and M. G. Rossmann.** 2006. West Nile virus in complex with the Fab fragment of a neutralizing monoclonal antibody. Proc Natl Acad Sci U S A **103:**12400-4.
27. **Khanam, S., B. Etemad, N. Khanna, and S. Swaminathan.** 2006. Induction of neutralizing antibodies specific to dengue virus serotypes 2 and 4 by a bivalent antigen composed of linked envelope domains III of these two serotypes. Am J Trop Med Hyg **74:**266-77.
28. **Khanam, S., N. Khanna, and S. Swaminathan.** 2006. Induction of neutralizing antibodies and T cell responses by dengue virus type 2 envelope domain III encoded by plasmid and adenoviral vectors. Vaccine **24:**6513-25.
29. **Kiermayr, S., K. Stiasny, and F. X. Heinz.** 2009. Impact of quaternary organization on the antigenic structure of the tick-borne encephalitis virus envelope glycoprotein E. J Virol **83:**8482-91.
30. **Kuhn, R. J., W. Zhang, M. G. Rossmann, S. V. Pletnev, J. Corver, E. Lenches, C. T. Jones, S. Mukhopadhyay, P. R. Chipman, E. G. Strauss, T.**

S. Baker, and J. H. Strauss. 2002. Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. Cell **108:**717-25.

31. **Lai, C. Y., W. Y. Tsai, S. R. Lin, C. L. Kao, H. P. Hu, C. C. King, H. C. Wu, G. J. Chang, and W. K. Wang.** 2008. Antibodies to envelope glycoprotein of dengue virus during the natural course of infection are predominantly cross-reactive and recognize epitopes containing highly conserved residues at the fusion loop of domain II. J Virol **82:**6631-43.
32. **Lee, J. W., J. J. Chu, and M. L. Ng.** 2006. Quantifying the specific binding between West Nile virus envelope domain III protein and the cellular receptor alphaVbeta3 integrin. J Biol Chem **281:**1352-60.
33. **Lindenbach, B. D., Thiel, H.-J., Rice, C.M.** 2007. Flaviviridae: the viruses and their replication, p. 1101-1152. *In* D. M. Knipe, Howley, P.M. (ed.), Fields Virology, 5th ed. Lippincott Williams & Wilkins Co., Philadelphia, PA., Philadelphia.
34. **Lok, S. M., V. Kostyuchenko, G. E. Nybakken, H. A. Holdaway, A. J. Battisti, S. Sukupolvi-Petty, D. Sedlak, D. H. Fremont, P. R. Chipman, J. T. Roehrig, M. S. Diamond, R. J. Kuhn, and M. G. Rossmann.** 2008. Binding of a neutralizing antibody to dengue virus alters the arrangement of surface glycoproteins. Nat Struct Mol Biol **15:**312-7.
35. **Mason, P. W., J. M. Dalrymple, M. K. Gentry, J. M. McCown, C. H. Hoke, D. S. Burke, M. J. Fournier, and T. L. Mason.** 1989. Molecular characterization of a neutralizing domain of the Japanese encephalitis virus structural glycoprotein. J Gen Virol **70 (Pt 8):**2037-49.
36. **Modis, Y., S. Ogata, D. Clements, and S. C. Harrison.** 2003. A ligand-binding pocket in the dengue virus envelope glycoprotein. Proc Natl Acad Sci U S A **100:**6986-91.
37. **Modis, Y., S. Ogata, D. Clements, and S. C. Harrison.** 2005. Variable surface epitopes in the crystal structure of dengue virus type 3 envelope glycoprotein. J Virol **79:**1223-31.
38. **Mukhopadhyay, S., B. S. Kim, P. R. Chipman, M. G. Rossmann, and R. J. Kuhn.** 2003. Structure of West Nile virus. Science **302:**248.
39. **Nelson, S., C. A. Jost, Q. Xu, J. Ess, J. E. Martin, T. Oliphant, S. S. Whitehead, A. P. Durbin, B. S. Graham, M. S. Diamond, and T. C. Pierson.**

2008. Maturation of West Nile virus modulates sensitivity to antibody-mediated neutralization. PLoS Pathog **4:**e1000060.

40. **Nybakken, G. E., C. A. Nelson, B. R. Chen, M. S. Diamond, and D. H. Fremont.** 2006. Crystal structure of the West Nile virus envelope glycoprotein. J Virol **80:**11467-74.
41. **Nybakken, G. E., T. Oliphant, S. Johnson, S. Burke, M. S. Diamond, and D. H. Fremont.** 2005. Structural basis of West Nile virus neutralization by a therapeutic antibody. Nature **437:**764-9.
42. **Oliphant, T., M. Engle, G. E. Nybakken, C. Doane, S. Johnson, L. Huang, S. Gorlatov, E. Mehlhop, A. Marri, K. M. Chung, G. D. Ebel, L. D. Kramer, D. H. Fremont, and M. S. Diamond.** 2005. Development of a humanized monoclonal antibody with therapeutic potential against West Nile virus. Nat Med **11:**522-30.
43. **Oliphant, T., G. E. Nybakken, S. K. Austin, Q. Xu, J. Bramson, M. Loeb, M. Throsby, D. H. Fremont, T. C. Pierson, and M. S. Diamond.** 2007. Induction of epitope-specific neutralizing antibodies against West Nile virus. J Virol **81:**11828-39.
44. **Oliphant, T., G. E. Nybakken, M. Engle, Q. Xu, C. A. Nelson, S. Sukupolvi-Petty, A. Marri, B. E. Lachmi, U. Olshevsky, D. H. Fremont, T. C. Pierson, and M. S. Diamond.** 2006. Antibody recognition and neutralization determinants on domains I and II of West Nile Virus envelope protein. J Virol **80:**12149-59.
45. **Peeters, C. C., A. M. Tenbergen-Meekes, J. T. Poolman, M. Beurret, B. J. Zegers, and G. T. Rijkers.** 1991. Effect of carrier priming on immunogenicity of saccharide-protein conjugate vaccines. Infect Immun **59:**3504-10.
46. **Pierson, T. C., and M. S. Diamond.** 2008. Molecular mechanisms of antibody-mediated neutralisation of flavivirus infection. Expert Rev Mol Med **10:**e12.
47. **Pierson, T. C., D. H. Fremont, R. J. Kuhn, and M. S. Diamond.** 2008. Structural insights into the mechanisms of antibody-mediated neutralization of flavivirus infection: implications for vaccine development. Cell Host Microbe **4:**229-38.
48. **Plotkin, S. A., W. A. Orenstein, and P. A. Offit.** 2008. Vaccines 5th edition. (Saunders).

49. **Rey, F. A., F. X. Heinz, C. Mandl, C. Kunz, and S. C. Harrison.** 1995. The envelope glycoprotein from tick-borne encephalitis virus at 2 A resolution. Nature **375:**291-8.
50. **Roehrig, J. T.** 2003. Antigenic structure of flavivirus proteins. Adv Virus Res **59:**141-75.
51. **Roehrig, J. T., R. A. Bolin, and R. G. Kelly.** 1998. Monoclonal antibody mapping of the envelope glycoprotein of the dengue 2 virus, Jamaica. Virology **246:**317-28.
52. **Sanchez, M. D., T. C. Pierson, M. M. Degrace, L. M. Mattei, S. L. Hanna, F. Del Piero, and R. W. Doms.** 2007. The neutralizing antibody response against West Nile virus in naturally infected horses. Virology **359:**336-48.
53. **Simmons, M., K. R. Porter, C. G. Hayes, D. W. Vaughn, and R. Putnak.** 2006. Characterization of antibody responses to combinations of a dengue virus type 2 DNA vaccine and two dengue virus type 2 protein vaccines in rhesus macaques. J Virol **80:**9577-85.
54. **Spohn, G., G. T. Jennings, B. E. Martina, I. Keller, M. Beck, P. Pumpens, A. D. Osterhaus, and M. F. Bachmann.** 2010. A VLP-based vaccine targeting domain III of the West Nile virus E protein protects from lethal infection in mice. Virol J **7:**146.
55. **Steel, J., A. C. Lowen, T. Wang, M. Yondola, Q. Gao, K. Haye, A. Garcia-Sastre, and P. Palese.** 2010. Influenza virus vaccine based on the conserved hemagglutinin stalk domain. MBio **1**.
56. **Stiasny, K., H. Holzmann, and F. X. Heinz.** 2009. Characteristics of antibody responses in tick-borne encephalitis vaccination breakthroughs. Vaccine **27:**7021-6.
57. **Stiasny, K., S. Kiermayr, H. Holzmann, and F. X. Heinz.** 2006. Cryptic properties of a cluster of dominant flavivirus cross-reactive antigenic sites. J Virol **80:**9557-68.
58. **Sukupolvi-Petty, S., S. K. Austin, W. E. Purtha, T. Oliphant, G. E. Nybakken, J. J. Schlesinger, J. T. Roehrig, G. D. Gromowski, A. D. Barrett, D. H. Fremont, and M. S. Diamond.** 2007. Type- and subcomplex-specific neutralizing antibodies against domain III of dengue virus type 2 envelope protein recognize adjacent epitopes. J Virol **81:**12816-26.

59. **Throsby, M., C. Geuijen, J. Goudsmit, A. Q. Bakker, J. Korimbocus, R. A. Kramer, M. Clijsters-van der Horst, M. de Jong, M. Jongeneelen, S. Thijsse, R. Smit, T. J. Visser, N. Bijl, W. E. Marissen, M. Loeb, D. J. Kelvin, W. Preiser, J. ter Meulen, and J. de Kruif.** 2006. Isolation and characterization of human monoclonal antibodies from individuals infected with West Nile Virus. J Virol **80:**6982-92.

60. **Valdes, I., L. Hermida, L. Gil, L. Lazo, J. Castro, J. Martin, L. Bernardo, C. Lopez, O. Niebla, T. Menendez, Y. Romero, J. Sanchez, M. G. Guzman, and G. Guillen.** 2010. Heterologous prime-boost strategy in non-human primates combining the infective dengue virus and a recombinant protein in a formulation suitable for human use. Int J Infect Dis **14:**e377-83.

61. **Virgin, H. W., and B. D. Walker.** 2010. Immunology and the elusive AIDS vaccine. Nature **464:**224-31.

62. **Wahala, W. M., A. A. Kraus, L. B. Haymore, M. A. Accavitti-Loper, and A. M. de Silva.** 2009. Dengue virus neutralization by human immune sera: role of envelope protein domain III-reactive antibody. Virology **392:**103-13.

63. **Wu, K. P., C. W. Wu, Y. P. Tsao, T. W. Kuo, Y. C. Lou, C. W. Lin, S. C. Wu, and J. W. Cheng.** 2003. Structural basis of a flavivirus recognized by its neutralizing antibody: solution structure of the domain III of the Japanese encephalitis virus envelope protein. J Biol Chem **278:**46007-13.

64. **Wu, S. C., C. H. Yu, C. W. Lin, and I. M. Chu.** 2003. The domain III fragment of Japanese encephalitis virus envelope protein: mouse immunogenicity and liposome adjuvanticity. Vaccine **21:**2516-22.

65. **Zhang, W., P. R. Chipman, J. Corver, P. R. Johnson, Y. Zhang, S. Mukhopadhyay, T. S. Baker, J. H. Strauss, M. G. Rossmann, and R. J. Kuhn.** 2003. Visualization of membrane protein domains by cryo-electron microscopy of dengue virus. Nat Struct Biol **10:**907-12.

FIGURES

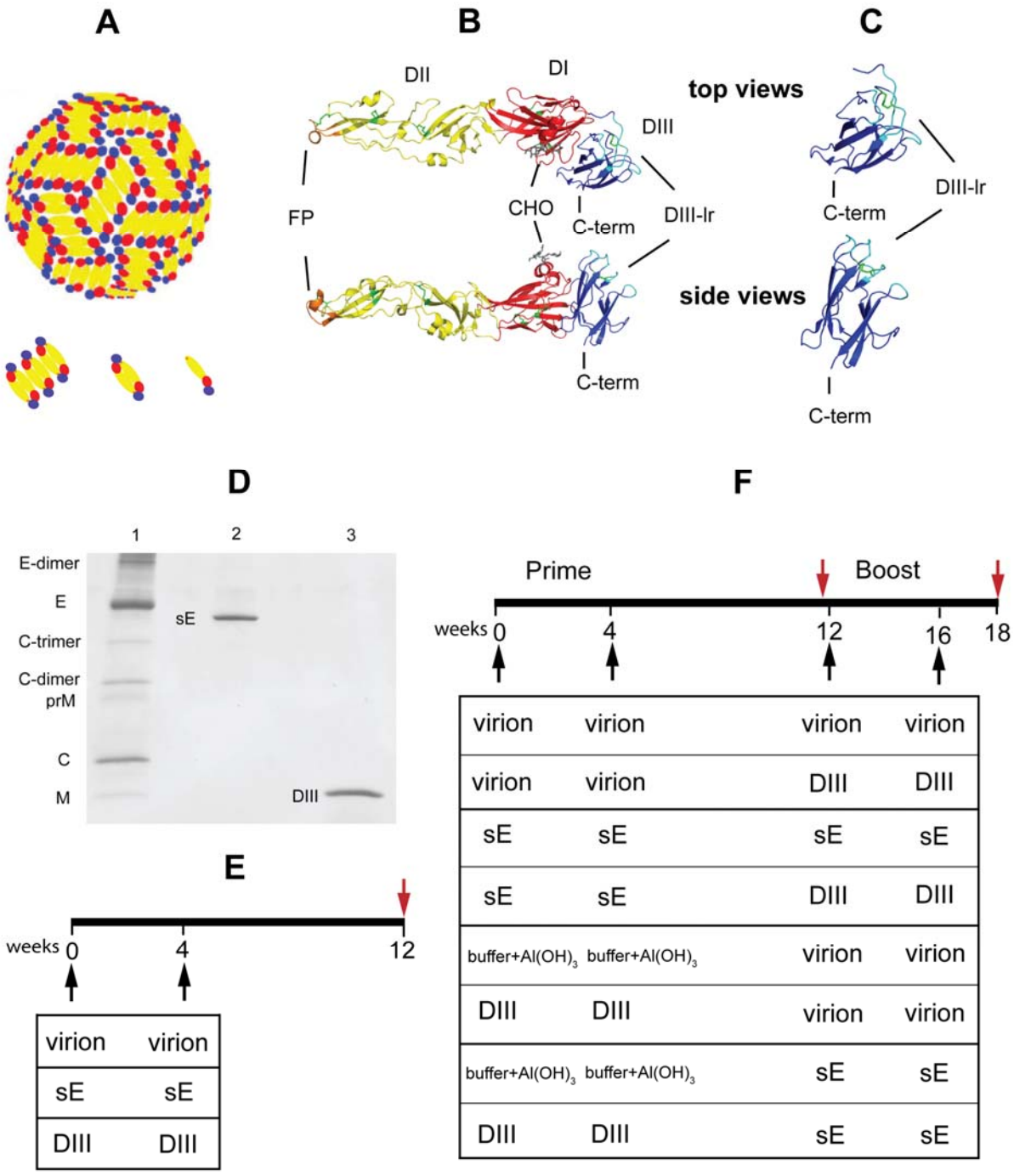

FIG 1. Immunogens and immunization schedules. (A) Schematic model of a flavivirus virus particle based on cryo-EM reconstructions of dengue and WN viruses (30, 38). The viral surface is covered by a densely packed shell consisting of 90 copies of E protein dimers which are arranged in a herring bone-like lattice, consisting of 30 rafts of three E dimers. One of these rafts, an E dimer and an E monomer are depicted separately at the bottom of this panel. (B) Ribbon diagrams (top and side view) of the WN virus soluble E protein monomer (40), (pdb ID 2HG0).

(C) Ribbon diagrams (top and side view) of the WN virus DIII (40), (pdb ID 2HG0). In panels A, B and C, DI, DII and DIII of E are shown in red, yellow and blue, respectively. The fusion peptide (FP) loop at the tip of DII is highlighted in orange, the surface exposed DIII lateral ridge epitope (DIII-lr) in cyan, disulphide bridges in green, and the carbohydrate side chain (CHO) in grey. (D) Analysis of the immunogens used in the study by SDS-PAGE (15%gel) and Coomassie staining. Lane 1: Formalin inactivated purified WN virus. Oligomeric bands of C and E are a result of protein-crosslinks as a result of formalin treatment. Lane 2: Recombinant WN virus sE. Lane 3: Recombinant WN virus DIII. (E) Schematic of the immunization schedule with virion (inactivated purified virus), sE, and DIII. With each of these antigens, a total of 30 mice (divided into groups of 10) were immunized with 2 doses at an interval of 4 weeks. Blood samples were taken 8 weeks after the second immunization and the sera of each group of 10 mice were pooled for further analyses. (F) Schematic of the immunization schedule for 8 different prime-boost regimens. The schedule consisted of two primary immunizations at an interval of four weeks followed by two booster immunizations after eight weeks, again at an interval of four weeks. For each regimen 10 mice were used. Blood samples were taken eight weeks after the second primary immunization as well as two weeks after the second booster immunization and pooled for further analyses.

In panels E and F, black arrows indicate time points of immunization and red arrows time points of blood sampling.

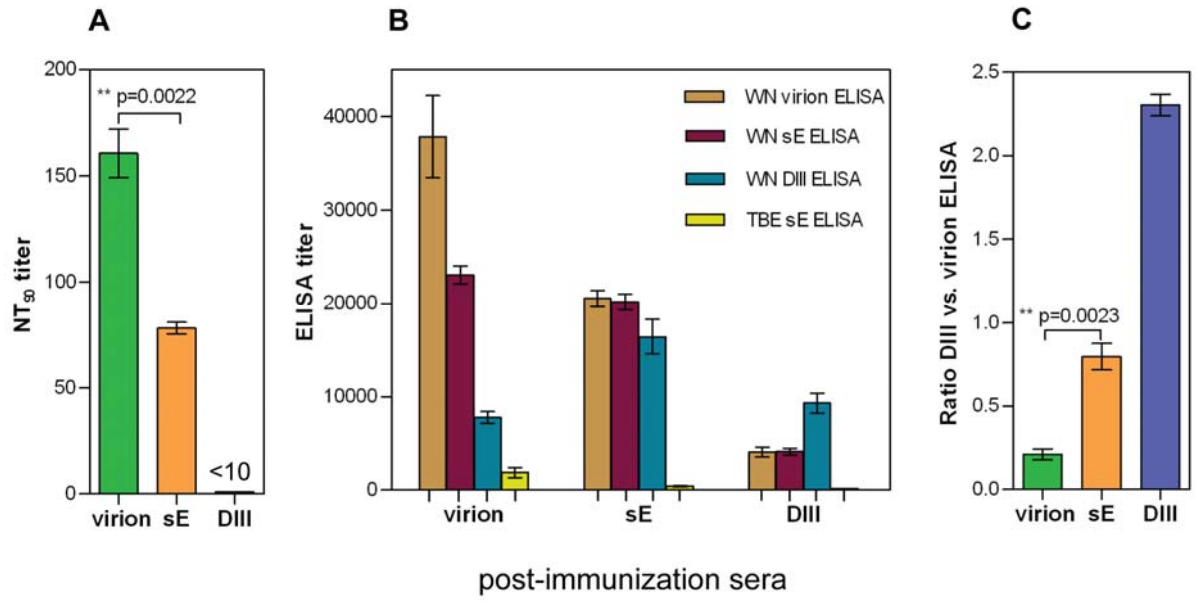

FIG 2. Serum antibody titers obtained after immunization with virion, sE and DIII. (A) Virus neutralization titers (NT_{90}). (B) ELISA titers against different antigens, as indicated by the colour codes in the inset. (C) Relative contents of DIII antibodies, displayed as the ratio of DIII- versus virion-specific ELISA titers. The data presented were derived from the analysis of pooled sera obtained from three groups of ten mice per antigen. The figure displays the means of the results obtained with these three serum pools. The error bars represent the standard errors of the means.

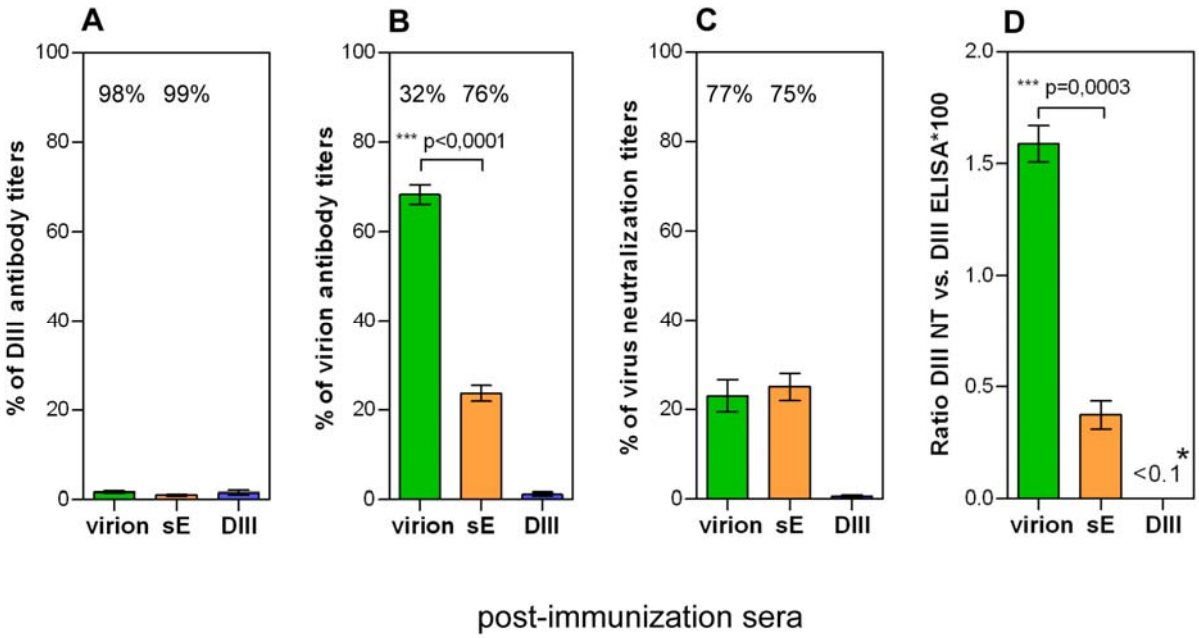

FIG 3. ELISA and NT analysis of virion, sE and DIII post-immunization sera after depletion with DIII. (A) Percent DIII-reactive antibodies in post-depletion sera, determined by DIII ELISA. (B) Percent virion-reactive antibodies in post-depletion sera, determined by virion ELISA. (C) Percent virus neutralizing antibodies in post-depletion sera, determined by neutralization assay. Results are expressed as percent of the titers before depletion. The DIII induced neutralizing antibody titer was <10 already before depletion (see Fig.2A). The numbers above the bars in A, B, and C indicate the calculated percentage of the contribution of DIII antibodies to total reactivity. (D) Ratio of DIII-associated neutralizing titers versus DIII-specific ELISA titers x100, as a measure of the specific neutralizing activity and thus the functional quality of DIII-reactive antibodies. The DIII-associated neutralizing titers were deduced from the data in Fig. 2A and 3C and calculated to be 123 and 58 for virion and sE sera, respectively.

*The NT_{90}-titer of DIII-induced antibodies was <10 and therefore no exact ratio could be calculated. The error bars represent the standard errors of the means obtained in three independent determinations.

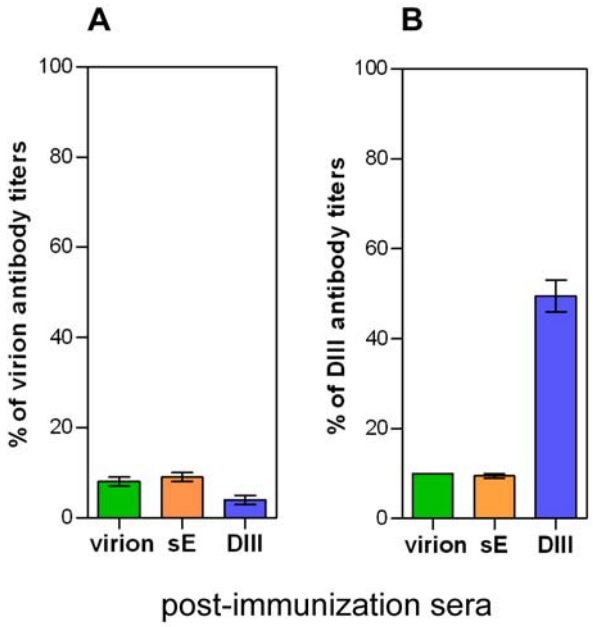

FIG 4. ELISA analysis of virion, sE, and DIII post-immunization sera after depletion with virion. (A) Percent virion-reactive antibodies in post-depletion sera, determined by virion ELISA. (B) Percent DIII-reactive antibodies in post-depletion sera determined by DIII ELISA. Results are expressed as percent of the titers before depletion. The error bars represent the standard errors of the means obtained in three independent determinations.

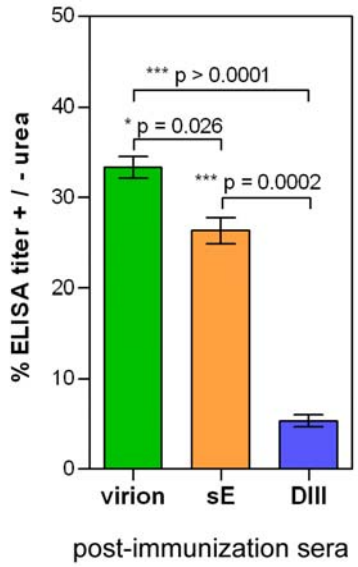

FIG. 5. Relative avidities of virion, sE, and DIII post-immunization sera determined in virion ELISA +/- urea. The error bars represent the standard errors of the means obtained in three independent determinations.

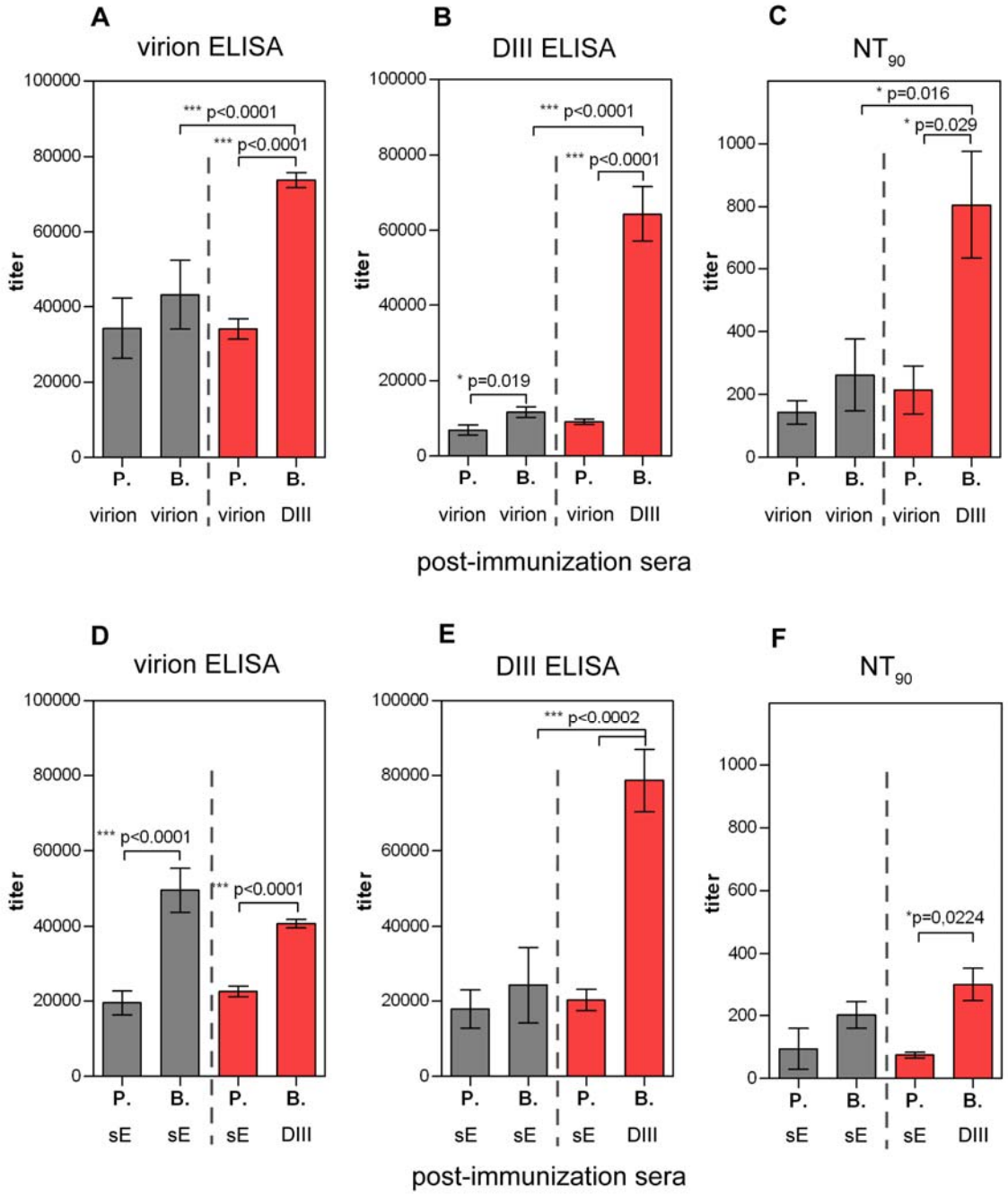

FIG 6. ELISA and NT analysis of post-immunization sera using virion or sE for priming and DIII for boosting. Panels A to C: Priming (P.) with virion and boosting (B.) with DIII. Panels D to F: Priming (P.) with sE and boosting (B.) with DIII. Post-immunization sera were analyzed in virion ELISA (A and D), DIII-ELISA (B and E) and NT (C and F). The error bars represent the standard errors of the means obtained in at least three independent determinations.

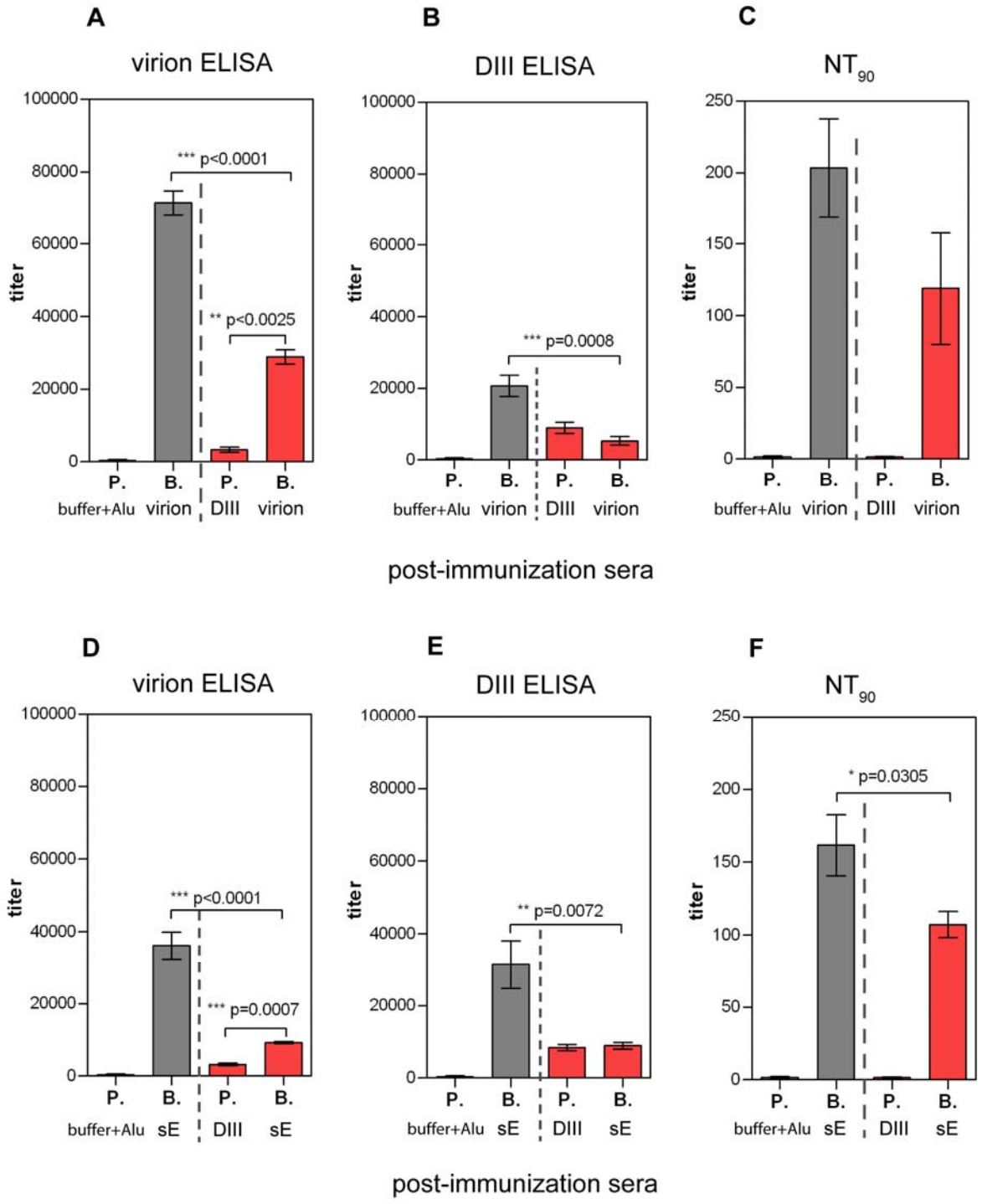

FIG 7. ELISA and NT analysis of post-immunization sera using DIII for priming and virion or sE for boosting. Panels A to C: Priming (P.) with DIII and boosting (B.) with virion. Panels D to F: Priming (P.) with DIII and boosting (B.) with sE. Post-immunization sera were analyzed in virion ELISA (A and D), DIII-ELISA (B and E) and NT (C and F).

The error bars represent the standard errors of the means obtained in at least three independent determinations.

4. Part II: Unpublished Data

4.1 Materials and Methods

4.1.1 Production of inactivated TBE and WN viruses

Production of highly purified viruses was carried out essentially as described in (39). In brief, primary chicken embryo fibroblasts were infected with WN virus, strain NY99 (GenBank# AF196835) or TBE virus strain Neudörfl (GenBank# U27495). The cell supernatant was harvested 24 hours p.i., clarified by centrifugation and inactivated with formalin (1:2000) for 24 hrs at 37°C. The inactivated virus was concentrated by ultracentrifugation and purified by rate zonal followed by equilibrium sucrose density gradient centrifugation.

4.1.2 Cloning, expression and purification of recombinant soluble E proteins

4.1.2.1 Cloning of recombinant sE

Prior to PCR of prM-sE from TBE virus strain Neudörfl, the Age I restriction site in the E protein of the RSP template (2) was mutated by the introduction of a silent point mutation (A-G) at position 960 in the E protein nucleotide sequence by site directed mutagenesis (invitrogen) using 5`-GCTCCTGTGTTTGGCG CCGGTTTACGCTTC-3` and 5`-GCCAAACACAGGAGCACAACAAGAACGGCG-3` as forward and reverse primers, respectively. DNA cassettes, encoding prM and soluble, truncated forms (first 400 amino acids) of the E proteins (sE) from TBE and WN viruses were amplified using the respective RSP templates with the following primers:

TBE sE His:

- forward: 5'- TTTTAAGAGATCTGCAACGGTGAGGAAAGAAAG-3'
- reverse: 5'- ATATAACCGGGTCCGATGCTGCTCCCTTTTTG-3'

WN sE:

- forward: 5'- AAGAGATCTGGTACCCTCTCTAACTTC-3'
- reverse: 5'- ATATAACCGGTTCATCAAGACTTGTGCCAATGGTG-3'

WN sE-His:

- forward:5'- AAGAGATCTGGTACCCTCTCTAACTTC-3'
- reverse: 5'- ATATAACCGGTTCATCAAGACTTGTGCCAATGGTG-3'

The PCR products were subjected to agarose gel electrophoresis, the appropriate DNA fragments were purified from the gel, digested using Bgl II and Age I (New England Biolabs) restriction enzymes and ligated into the corresponding restriction sites of the dephophorylated expression vector pMTBiP/V5-His (Invitrogen) using the LigaFast Rapid DNA Ligation System (Promega) according to the manufacturers' protocol. Correct sequences of the inserts in the recombinant DNA plasmids were verified by DNA sequencing.

4.1.2.2 Stable transfection of S2 cells and expression of recombinant sE proteins

Stable transfection of Drosophila Schneider 2 (S2) cells was performed essentially as recommended by Invitrogen. Briefly, the recombinant expression plasmids were co-transfected with a blasticidin selection plasmid. After transfection, the cells were grown in Schneider`s Drosophila medium (Gibco) containing 10% FCS (PAA), Penicillin, Streptomycin, Amphotericin B (Invitrogen) and supplemented with 25µg/ml blasticidin (Invitrogen) to select for stable transfected cells over a period of two weeks and then adapted to protein free medium (Insect Xpress) as recommended by the manufacturer (Lonza).

For the production of the recombinant sE proteins, $1.5*10^6$cells/ml were seeded into protein free medium supplemented with 10 µg/ml blasticidin (Invitrogen). Shaking cultures were maintained at 100rpm at 28°C and the expression of the recombinant sE proteins was induced by adding 500 mM Cu^{2+}. The cell culture supernatant was harvested 7 to 11 days post-induction by centrifugation, filtered through a 0.2 µm filter (Millipore) and stored at -20°C.

4.1.2.3 Purification of recombinant sE proteins

For purification, the cell culture supernatants containing recombinant sE proteins from TBE and WN viruses, were subjected to immunoaffinity chromatography using the broadly flavivirus cross-reactive mouse monoclonal antibody 4G2 (ATCC# HB-112), which was coupled to a sepharose column via N-hydroxysuccinimide chemistry (GE healthcare). The bound recombinant proteins were eluted by 20 mM glycine, pH 2.5. Peak fractions were back-neutralized immediately to pH 8.0 with 2 M Tris-Cl pH 9.5, buffer exchanged to TAN buffer (0,05 M triethanolamin 0,1 M NaCl, pH 8.0) by ultrafiltration and stored at -80°C.

4.1.3 Cloning, expression and purification of recombinant DIII proteins

4.1.3.1 Cloning of recombinant DIII

DNA fragments encoding TBE virus (strain Neudörfl) E protein amino acids 302-394 and WN virus (strain NY99) E protein amino acids 300-399 were amplified by PCR from the respective RSP clones as templates (2), using HFII polymerase (Clontech) using the following primers:

TBE DIII:

- Forward: 5`- GGTATTGAGGGTCGCCTTACGTACACAATGTGT-3`
- Reverse: 5`-AGAGGAGAGTTAGAGCCTCATCATTGGAACCATTGATGACT-3`

TBE DIII-His:

- Forward: 5`-GGTATTGAGGGTCGCCTTACGTACACAATGTGT-3`
- Reverse:5`-AGAGGAGAGTTAGAGCCTCATCAGTGGTGGTGGTGGTGGT GTTGGAACCATTGATGACTC-3`

WNV DIII:

- forward 5`-GGTATTGAGGGTCGCACAACCTATGGCGTCTGTTC-3`
- reverse: 5`-AGAGGAGAGTTACCTCATCACTTGTGCCAATGGTGATTG-3`

WNV DIII-His

- Forward: 5`-GGTATTGAGGGTCGCACAACCTATGGCGTCTGTTC-3`
- reverse:5`-AGAGGATTAGAGCCTCATCAGTTGTTGTTGTTGTTGTGCTT

GTGCCAATGGTGATGG-3`

The PCR products were subjected to agarose gel electrophoresis, the appropriate DNA fragments were purified from the gel, treated with T4 DNA polymerase as recommended by Novagen and cloned into the pET 32a Xa/LIC expression vector by ligation independent cloning, according to the manufacturer's instructions (Novagen). Correct sequences of the clones were verified by DNA sequencing. In the case of the vector encoding the DIII-His fusion proteins, the DNA sequences encoding the N-terminal His-tag were deleted by site-directed mutagenesis (Invitrogen) and the correct sequences were verified by DNA sequencing (Fig 4.1).

4.1.3.2 Expression of DIII-thioredoxin fusionproteins in E.coli

The recombinant DIII expression plasmids were transformed into E.coli strain BL21 (Novagen). 500ml of fresh Luria Broth medium, supplemented with 100µg/ml ampicillin (Sigma), were inoculated with 20ml of an over night culture (o/n) and grown at 37°C until the OD_{600} reached 0.5-0.6. Expression of the fusion proteins was induced by the addition of Isopropyl-ß-D thiogalactopyranoside (IPTG, Sigma) to a final concentration of 0.5 mM for 4h at 30°C. The cells were harvested by centrifugation at 16,000g for 10 minutes at 4°C, lysed with Bug Buster Protein Extraction Reagent (Novagen) and the lysate was centrifuged at 16,000g at 4°C for 30 min. The supernatant was filtered through a 0.2µm filter and used for purification of DIII.

4.1.3.3 Purification of recombinant DIII proteins

The supernatant of the lysate of DIII producing bacteria was applied onto chelating sepharose columns (GE Healthcare) loaded with Ni^{2+}. The column was washed with buffer containing 20mM imidazole equilibrated with factor Xa cleavage buffer and thioredoxin was cleaved off by proteolytic cleavage over night at room temperature with 90U/ml of factor Xa (Novagen). After cleavage, DIII proteins (used as immunogens in mice) were eluted from the column by washing with buffer, leaving

the thioredoxin fusion partner bound to the column (Fig. 4.1A). In the case of DIII-His used for immunological assays, the thioredoxin fusion partner was cleaved off from DIII-His by factor Xa and eluted with wash buffer, while the DIII-His protein remained bound to the column. DIII-His was then eluted by a linear imidazole gradient (0-500mM imidazole) (Fig. 4.1B).

The DIII-protein containing solution was buffer exchanged to 20mM triethanolamin, pH 8.0, by ultra-filtration (MWCO=3000Da, Sartorius), applied onto Sepharose Q anion exchange chromatography columns (GE healthcare Life Sciences), and eluted by a continuous salt gradient (0-1M NaCl). The DIII preparations were buffer exchanged to TAN, pH 8.0, using ultrafiltration and stored at -80°C.

4.1.4 Protein quantification assay

The concentrations of purified recombinant proteins were determined by BCA (bicinchoninic acid) protein assay (Pierce) according to the manufacturer's instructions.

4.1.5 S-alkylation of recombinant DIII and sE proteins

Purified recombinant sE and DIII proteins in TAN pH 8.0 were reduced with 100mM dithiothreitol (DTT) in the presence of 2% SDS, for 1h at 65°C. Free -SH groups in the cysteines were alklyated (S-alkylation) by incubation with 300mM iodoacetic acid (dissolved in 1M NaOH acid, pH 8.0) for 30min at room temperature. The reaction was stopped by adding DTT. The buffer was exchanged to TAN, pH 8.0, using PD-10 desalting columns (GE Healthcare).

4.1.6 Western blot analysis

100 ng of purified recombinant proteins were subjected to 15% SDS-PAGE according to Laemmli (52). The polyvinyldifluoride (PVDF, BioRad) membrane was soaked in methanol (Merck) and then equilibrated together with the gel and the

blotting paper in blotting buffer (48 mM Tris, 39 mM Glycine, 1.3 mM SDS, 20% methanol). The protein was transferred for 90min at 15V onto the PVDF membrane in a semidry blotting apparatus (Bio Rad). The membrane was blocked with 1% bovine serum albumin (BSA) in PBS + 0.1% Tween 20 over night at 4°C. The primary antibody, diluted in blocking solution, was added for 2h at RT. The membrane was washed with distilled H_2O and incubated with the peroxidase labelled IgG-specific secondary antibody for 90 minutes at RT. The substrate reaction was carried out using Sigma Fast DAB Urea/H_2O_2 tablets (Sigma) dissolved in H_2O+ 10% Ni^{2+}.

4.1.7 Mass spectrometry

Mass spectrometry (MS) analyses were performed at TOPLAB GmbH, Martinsried Germany. Briefly, recombinant DIII proteins from TBE and WN viruses were alkylated and proteolytically digested under reducing and non-reducing conditions. The peptide fragments were subjected to Matrix-assisted laser desorption/ionization (MALDI) and Time of Flight (TOF) mass spectrometric analyses and disulfide bridged peptides were identified according to their molecular mass.

4.1.8 Chemical cross-linking of recombinant sE proteins

Purified recombinant soluble E proteins in TAN pH 8.0 were incubated with 10mM dimethylsuberimidate (DMS) for 30 minutes at room temperature, precipitated with trichloroacetic acid and analyzed by SDS-polyacrylamide gel electrophoresis.

4.1.9 Sedimentation analyses

5µg of purified recombinant sEs and sE dimers isolated from infectious TBE virions (79) were applied to 7-20% (wt/wt) continuous sucrose gradients in TAN buffer, pH 8.0, or 2-(N-morpholino)ethanesulfonic acid (MES) buffer, pH 6.0, containing 0.1% Triton X-100 and centrifuged for 20h at 38,000 rpm at 15°C in a

Beckman SW40 rotor. After centrifugation, the gradient was fractionated and the fractions were used for further analyses.

4.1.10 Quantitative sE protein-specific SDS-ELISA

Microtiter plates (Nunc, Roskilde, Denmark) were coated with TBE virus-specific serum (raised in guinea pigs) or purified broadly cross-reactive mab 4G2 in carbonate buffer, pH 9.6, over night at 4°C for the catching of TBE or WN virus sEs, respectively. Samples (cell culture supernatants of stably transfected and induced S2 cells or purified sEs) were incubated in the presence of 0.4% SDS for 30 min at 65°C. Dilutions of the samples in ELISA buffer (PBS pH 7.4, 2% Tween-20, 2% lamb serum) were then added to the plates and incubated for 90 minutes at 37°C. The plates were washed, incubated with TBE or WN virus-specific polyclonal rabbit sera for 1h at 37°C, washed again and incubated with peroxidase labelled anti-rabbit IgG antibody for another 1h at 37°C. For the detection of the bound enzyme conjugate, o-phenylenediamine dihydrochloride (OPD, Sigma) was added and the reaction was stopped with 2N H_2SO_4. The absorbance was measured at 490 nm. Calculation of protein concentrations in the samples was performed using a standard curve with the appropriate purified sE.

4.1.11 Mouse immunization experiments

(A) Dose finding using sE and DIII from TBE virus

Groups of four female 6 to 8 weeks old C57BL/6 mice (Charles River Laboratories, Kisslegg, Germany) were immunized sub-cutaneously with 1, 5 or 20 µg of soluble E purified from infectious TBE virions (79) or purified recombinant TBE DIII, each adsorbed to 0.2% $Al(OH)_3$ (Aluminum hydroxide gel, Sigma) in 100µl TAN buffer, pH 8.0. Mice were immunized three times at an interval of two weeks and blood samples were withdrawn using microvette 200 capillaries (Sarstedt) before each of the immunizations and two weeks after the third. The serum samples of each group were pooled for further analyses.

(B) Sequential prime-boost immunizations using WN virus antigens

Six to eight weeks old C57BL/6 mice (Charles River Laboratories) were immunized sub-cutaneously with inactivated whole virus (1µg), sE (5µg) or DIII (20µg) in 100µl TAN, of West Nile virus as described in the manuscript.

4.1.12 Virion ELISA

The virion ELISAs were carried out as described in the manuscript. For the analysis of sera from the dose-finding experiment, whole inactivated TBE virus was used as an antigen.

4.1.13 Blocking ELISA

Three-fold serial dilution of the recombinant TBE virus sE-His and sE isolated from infectious TBE virions (79) were incubated with a predefined concentration of monoclonal antibodies (mabs) for 90 minutes at 37°C. Mabs which were not blocked by sE were then detected by virion ELISA using TBE virus as antigen.

4.1.14 sE and DIII ELISAs:

Maxisorp microtiter plates (Nunc, Roskilde, Denmark) were coated with rabbit anti His-tag IgG antibody (QED Bioscience) in carbonate buffer, pH 9.6, over night at 4°C. Recombinant DIII-His or sE-His proteins of TBE and WN viruses were added at a concentration of 1 and 0.5µg/ml respectively, for 1h at 37°C. Three-fold serial dilutions (starting at 1:100) of polyclonal mouse sera were then added for 1h at 37°C. As negative controls, sera from naïve mice were included and used for cut-off determination. The bound antibodies were detected using peroxidase labelled goat anti-mouse immunoglobulin G (Pierce) for 1h at 37°C. Titration curves were established using Graph pad Prism 5 software and serum titers were defined at an absorbance of 490 nm and a cut-off of 0.35, as deduced from the analysis of negative control sera,

4.1.15 TBE and WN virus neutralization assays

TBE virus neutralization assay was carried out essentially as described in (94). In brief, twofold serial dilutions of mouse sera, starting at 1:10, were mixed with 25 plaque-forming units (pfu) of TBE virus incubated for 1h at 37°C, added to a BHK cell monolayer and incubated for three days at 37°C. The presence of virus in the supernatant was determined by a four-layer SDS-ELISA. The titer was defined as the reciprocal of the serum dilution resulting in a 50% reduction of in the absorbance in ELISA (NT_{50}).

The WN virus neutralization assay was carried out on Vero cells (ECACC #84113001) using WN strain NY99 as described in the manuscript.

4.1.16 Antibody depletion assay

Antibody depletion assays were performed as described in the manuscript. Recombinant TBE virus sE-His was used as an antigen for the depletion of broadly cross-reactive antibodies and DIII-His of TBE and WN viruses for the depletion of DIII-reactive antibodies.

4.2 Results

4.2.1 Production and characterisation of recombinant proteins of WN and TBE viruses

The goal of this part of the thesis was the production of highly pure and properly folded recombinant proteins for the use as immunogens in mice and as antigens in immunological assays for analyzing the mouse post-immunization sera. The following section describes the production and characterization of recombinant soluble, truncated E proteins (sE) and domain III (DIII) of the E protein both with and without a C-terminal His-tag, to be used for assays and for immunization, respectively. The immunization study was conducted with WN virus antigens and TBE virus antigens were used as controls.

4.2.1.1 Cloning, expression, purification and characterization of recombinant DIIIs

Studies with several flaviviruses had shown that properly folded DIII can be expressed in bacteria in high amounts (6, 69, 80, 102, 103). Therefore, a high-yield bacterial expression system (E.coli) was chosen for the production of recombinant DIII proteins. To promote the formation of the single disulfide bridge and to increase solubility, DIII was expressed as a fusion protein with thioredoxin (trx) (Fig. 4.1). In addition, the fusion protein contained a His-tag for affinity purification and a proteolytic cleavage site for the separation of DIII from the fusion partner (Fig. 4.1).

4.2.1.1.1 Cloning of DIII

For the production of DIII with and without a His-tag, two different cloning strategies were necessary (Fig. 4.1). The expression vector encoded two His-tags. One was located in the N-terminal part of the fusion protein which was cleaved off during the purification of the DIII proteins, and a second was located at the C-terminus, separated from the cloning site by a linker sequence (Fig. 4.1).

For the production of DIII without a His-tag, the C-terminal His-tag and the linker between the His-tag and the DIII insert had to be removed. This was accomplished by introducing a stop codon by PCR after the last DIII codon of the DIII insert (Fig. 4.1A).

For the production of His-tagged DIII two obstacles had to be overcome. First, the C-terminal vector-encoded His-tag was separated from the cloning site via a linker (indicated by a black bar in Fig 4.1B). To avoid the possibility of any cross reactivities of this linker in subsequent immunological assays, it was removed by the use of PCR primers, which were designed to introduce a His-tag followed by a stop codon immediately after the last DIII codon (Fig. 4.1B). Secondly, the N-terminal His-tag, next to the thioredoxin fusion partner, had to be removed, because the presence of both His-tags would cause problems during the Ni^{2+}-affinity purification of the DIII-His protein. Therefore the internal His-tag was removed by site directed mutagenesis after the DIII-His DNA fragment was cloned into the expression vector (Fig. 4.1B). Correct sequences of the recombinant DIII constructs were verified by DNA sequencing.

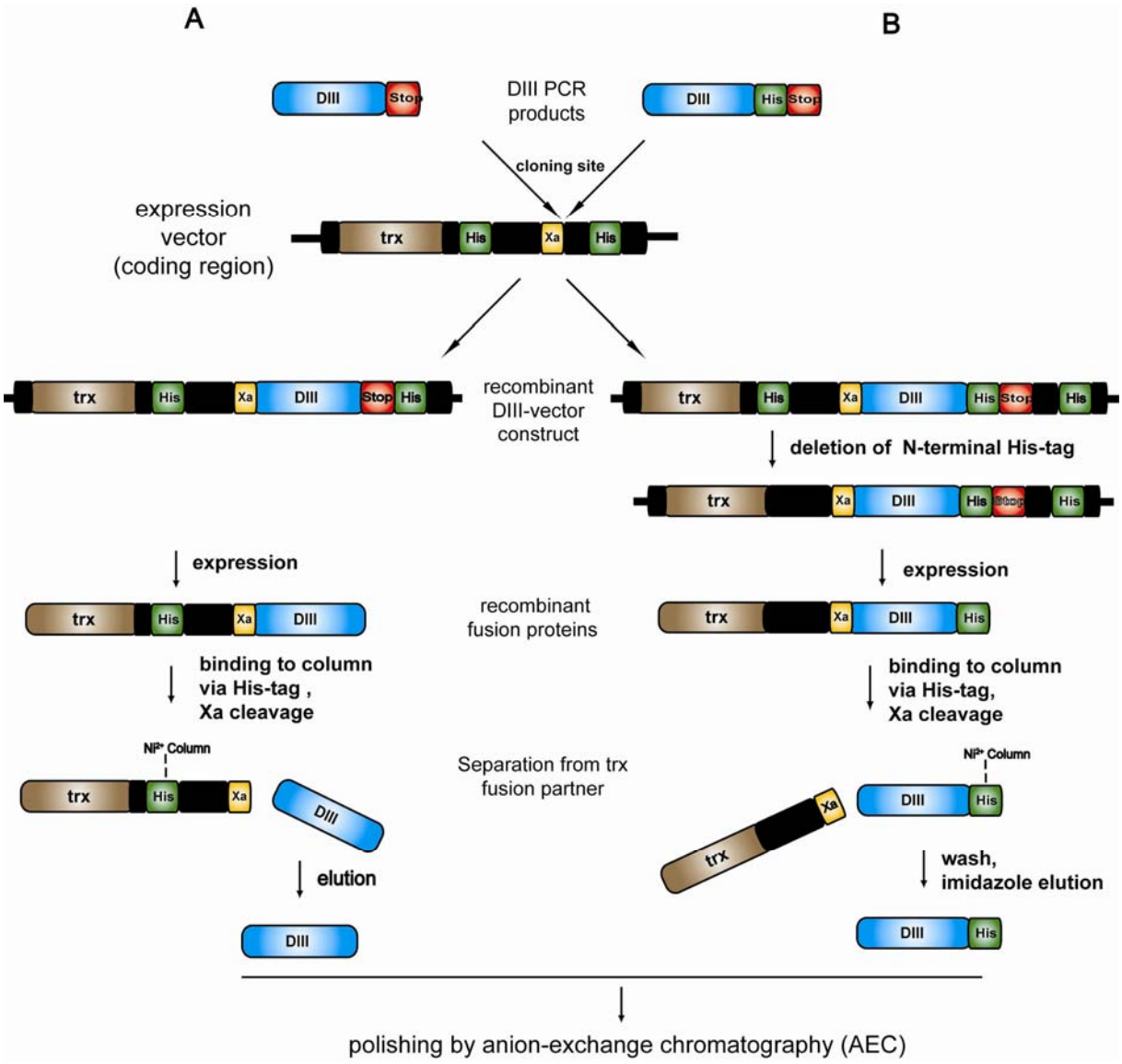

Fig. 4.1 Strategy for the cloning, expression and purification of recombinant DIII proteins.

(A) Cloning, expression and purification of DIII proteins used as immunogens in mice

(B) Cloning, expression and purification of DIII-His proteins used as antigens in immunological assays. DIII is colored in blue, stop codons (Stop) in red, His-tags in green, the thioredoxin fusion partner (trx) in brown, the proteolytic cleavage site (Xa) in yellow and linker sequences in black.

4.2.1.1.2 Expression of the trx-DIII fusion proteins in E.coli

For the expression of the fusion proteins, E.coli bacteria were transformed with the recombinant plasmids and expression was induced with IPTG. To analyze the expression of the fusion protein in the soluble and insoluble cell fractions, bacteria were harvested by centrifugation, lysed with detergent and the lysate was subjected to centrifugation at 10,000g for 10 minutes. Aliquots from the pellet (insoluble fraction), the supernatant (soluble fraction) as well as cells before lysis (total cellular protein) were subjected to SDS-PAGE and densitometric analysis to determine the distribution of the expressed fusion protein in the soluble and insoluble material. As shown in Fig.4.2 the fusion protein is present in the soluble as well as in the insoluble fraction, at a ratio of ~1:10, as determined by densitometric analysis.

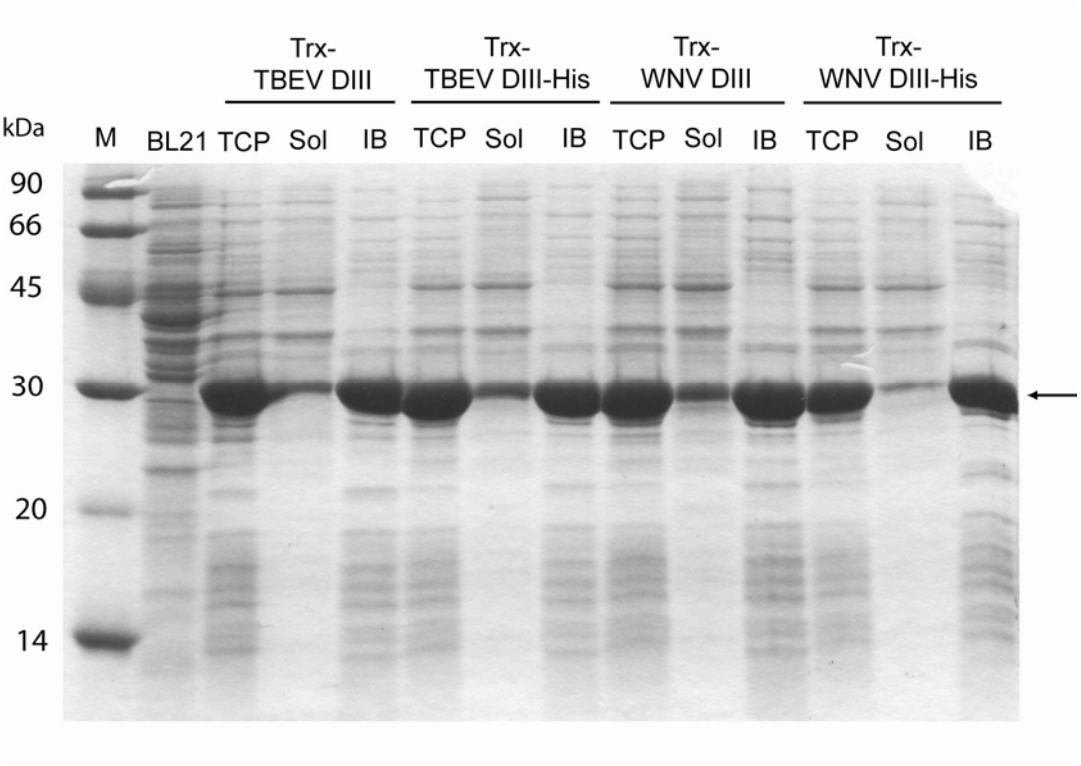

Fig. 4.2 SDS-PAGE and Coomassie staining of different cellular fractions of E.coli expressing trx-DIII fusion proteins.

Aliquots of total cellular protein (TCP), the soluble (sol) and the insoluble phase (inclusion bodies, IB) of cells expressing the trx-DIII fusion proteins of TBE and WN viruses were subjected to SDS-PAGE and Coomassie staining. The position of the trx-DIII fusion protein is indicated by an arrow. The soluble phase of E.coli not expressing the fusion protein (BL21) was used as a negative control. M: molecular weight markers.

4.2.1.1.3 Purification of recombinant DIII

Although most of the recombinant protein was found in the insoluble fraction, DIII was purified from the soluble fraction to avoid solubilisation of the protein from insoluble inclusion bodies with chaotropic reagents and the refolding of the denatured proteins. For purification, the soluble fraction of the bacterial lysate was applied onto a Ni^{2+} loaded column to bind the recombinant fusion proteins via the His-tag as indicated in Fig.4.1. As revealed by SDS-PAGE and densitometric analyses of the soluble phase and the column flow through, the fusion proteins bound almost quantitatively to the column (Fig 4.3C). The bound fusion proteins were proteolytically cleaved on the column by incubation with factor Xa. DIII without a His-tag was then eluted by a washing step while the fusion partner remained associated with the column via the internal His-tag (Fig. 4.1A). In contrast, His-tagged DIII remained bound to the column after Xa cleavage, and the cleaved-off thioredoxin fusion partner was removed by a washing step. DIII-His was then specifically eluted by an imidazole gradient (Fig. 4.1B). To determine recovery and purity, the eluted DIII preparations were subjected to SDS-PAGE and densitometric analyses of the stained protein bands. The recovery of the DIII proteins after the proteolytic digest was between 60 to 80% relative to the starting material (soluble fraction) (Fig. 4.3C). The obtained DIII proteins were insufficiently pure (see also sample in lane 4 of the SDS-PAGE analysis of a representative purification procedure of WN virus DIII-His) (Fig. 4.3A). To increase purity, the DIII proteins were subjected to a further round of purification using anion exchange chromatography. As shown in Fig. 4.3A and B, the purified DIII proteins migrated as single bands in SDS-gels and the recovery was between 40 to 55% (Fig. 4.3C).

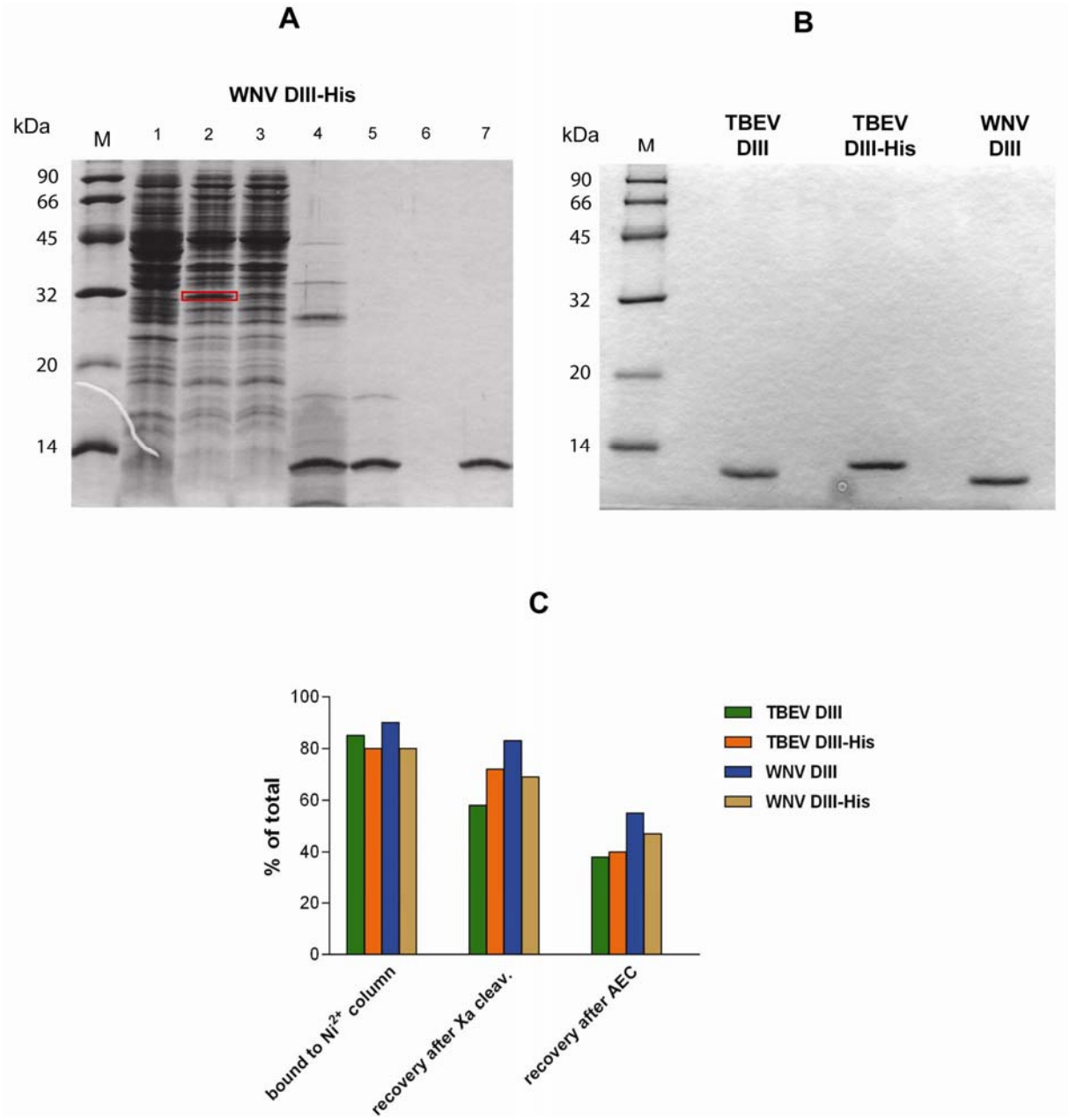

Fig. 4.3 Characterisation of the DIII purification process.

(A) SDS-PAGE and Coomassie staining of aliquots from successive steps of a representative DIII purification procedure (WN virus DIII-His). M = molecular weight marker; lane 1: soluble fraction of E.coli cells not expressing the thioredoxin-DIII fusion protein; lane 2: soluble fraction of E.coli expressing the thioredoxin-DIII fusion protein, indicated by a red rectangle; lane 3: Ni^{2+} column flow-through; lane 4: pool of eluted fractions after on-column proteolytic digest with factor Xa; lane 5: pool of elution fractions subjected to anion exchange chromatography (AEC) lane 6: AEC column wash after application; lane 7: pool of eluted DIII containing fractions from the AEC column;

(B) SDS-PAGE and Coomassie staining of purified TBE virus DIII, TBE virus DIII-His, and WN virus DIII proteins.

(C) Yield after successive purification steps, relative to the total amount of DIII in the starting material.

4.2.1.1.4 Characterization of the purified recombinant DIII proteins

The formation of the single disulfide bridge has been shown to be critical for proper folding of DIII (84). To asses the presence of the disulfide bond of purified DIII proteins, we first analyzed their migration behaviour by SDS-PAGE under reducing and non-reducing conditions. As a control, S-alkylated DIII was also included in the analysis (Fig. 4.4). For that purpose DIII was reduced and free –SH residues were chemically modified by the addition of an alkylating agent to avoid spontaneous reformation of the disulfide bridge (see Materials and Methods). As shown in figure 4.4A, TBE virus DIII protein exhibited a migration difference upon reduction and S-alkylation compared to the non-reduced sample, which is consistent with the presence of the disulphide bridge in this preparation. In contrast, using the TBE virus DIII as a control, WN virus DIII proteins did not show such a migratory difference upon reduction or S-alkylation (Fig. 4.4 B, C).

As a further test for proper folding, we employed a Western blot assay, under non-reducing conditions, using equal amounts of purified non-treated and S-alkylated DIIIs and conformation dependent DIII-specific monoclonal antibodies (mabs). These mabs lose reactivity upon reduction. As positive controls, TBE and WN virus specific polyclonal immune sera were used. As shown in Fig. 4.5, the polyclonal sera (KVIII, TBE virus-specific; KIS2, WN virus-specific) reacted both with the non-reduced as well as with the S-alkylated DIII proteins. In contrast, the conformation-dependent mabs B4 (TBE virus DIII-specific; (32)) and E16 (WN virus DIII-specific; (70)) reacted with the respective DIIIs under non-reducing conditions only, consistent with the presence of the disulfide bridge in these preparations.

In addition to the biochemical and immunological characterizations described above, mass spectrometry analyses were carried out to provide direct evidence for the presence of the disulphide bridge in the purified DIII preparations. The analysis was performed by a commercial protein analysis service (Toplab, Martinsried, Germany). After proteolytic digestion under reducing and non-reducing conditions, the resulting peptide fragments were analyzed by matrix-assisted-laser-desorption/ionization (MALDI) combined with time-of-flight (TOF) analysis. For all DIIIs analyzed, peptide fragments having a molecular mass corresponding to a disulfide bridged linear di-peptide were identified under non-reducing conditions. These were separated into two linear peptides of a distinct mass upon reduction,

providing direct evidence for the presence of the disulfide bridge in all DIII preparations (data not shown).

Taken together, these results suggest that the recombinant DIII proteins were obtained in a proper conformation.

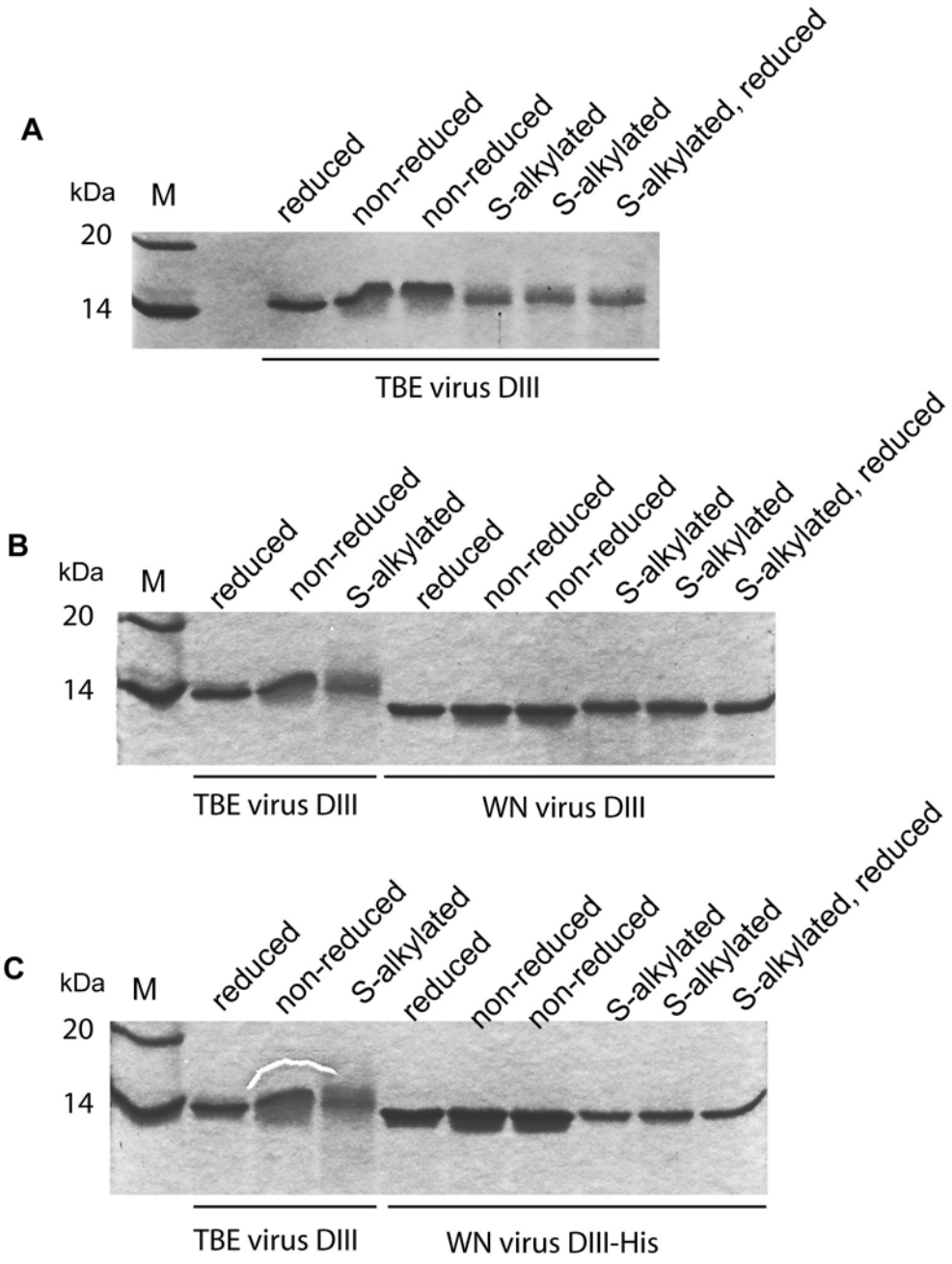

Fig. 4.4 SDS-PAGE and Coomassie staining of purified untreated and S-alkylated DIIIs under reducing and non-reducing conditions

(A) TBE virus DIII

(B) WN virus DIII

(C) WN virus DIII-His

In panels B and C, lanes 2-4 represent the TBE virus DIII as a control. M: molecular weight markers.

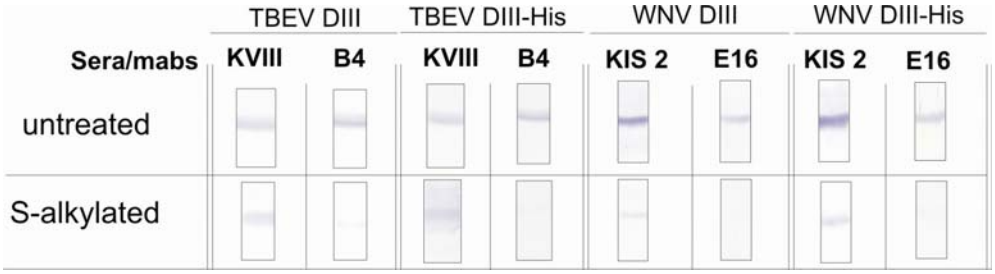

Fig. 4.5 Western blot of purified DIIIs under non-reducing conditions

Untreated and S-alkylated recombinant DIII proteins were subjected to SDS-PAGE and Western blotting under non-reducing conditions. Immunodetection was carried out with TBE (KVIII) and WN virus (KIS 2) -specific polyclonal sera (positive controls) and conformation-sensitive mabs B4 (TBE virus DIII-specific) and E16 (WN virus DIII-specific).

4.2.1.2 Cloning, expression, purification and characterization of recombinant sE proteins

The goal of this part of the thesis was to produce large amounts of highly pure and properly folded soluble, truncated forms of the envelope glycoprotein E (sE) of TBE and WN viruses using the Drosophila expression system. Analogous to the production of DIII, sE proteins were produced without a His-tag for immunization of mice and with a His-tag for the use as antigens in immunological assays.

4.2.1.2.1 Cloning of recombinant sE

As shown in Fig. 4.6, the expression vector encodes a drosophila-specific BIP signal sequence, which is necessary for targeting the proteins into the secretory pathway and their export into the cell culture supernatant, a so-called V5 epitope and a C-terminal His-tag. The V5 epitope is a 14 amino acid long tag which is not required for our study. Therefore, the Bgl II and Age I restriction sites were used for cloning, thus excising the DNA sequence coding for the V5 epitope (Fig. 4.6). PrM is believed to serve as a chaperone for the correct folding of E (62). Therefore, prM-sE encoding expression cassettes were amplified using RSP clones encoding the full-length prM and E proteins of the respective viruses (2) (Fig. 4.6) as templates. The E protein encoded by the TBE virus RSP construct contained an Age I restriction site

which was removed by the introduction of a silent mutation by site-directed mutagenesis, prior to amplification of the prM-sE cassette. Successful mutation was confirmed by DNA sequencing.

To generate sE without a His-tag for immunisation and with a His-tag for the use as an antigen in immunological assays, two different cloning strategies were employed, as indicated in Fig. 4.6. To clone sE without a His-tag, a stop codon was introduced by PCR immediately after the last codon of sE (Fig. 4.6A). To generate sE with a His-tag the prM-sE fragment was cloned in-frame with the vector-encoded His-tag (Fig. 4.6B). The amplified inserts were gel purified, treated with Bgl II and Age I restriction enzymes and cloned into the corresponding sites of the expression vector. Successful cloning and correct sequences were verified by DNA sequencing.

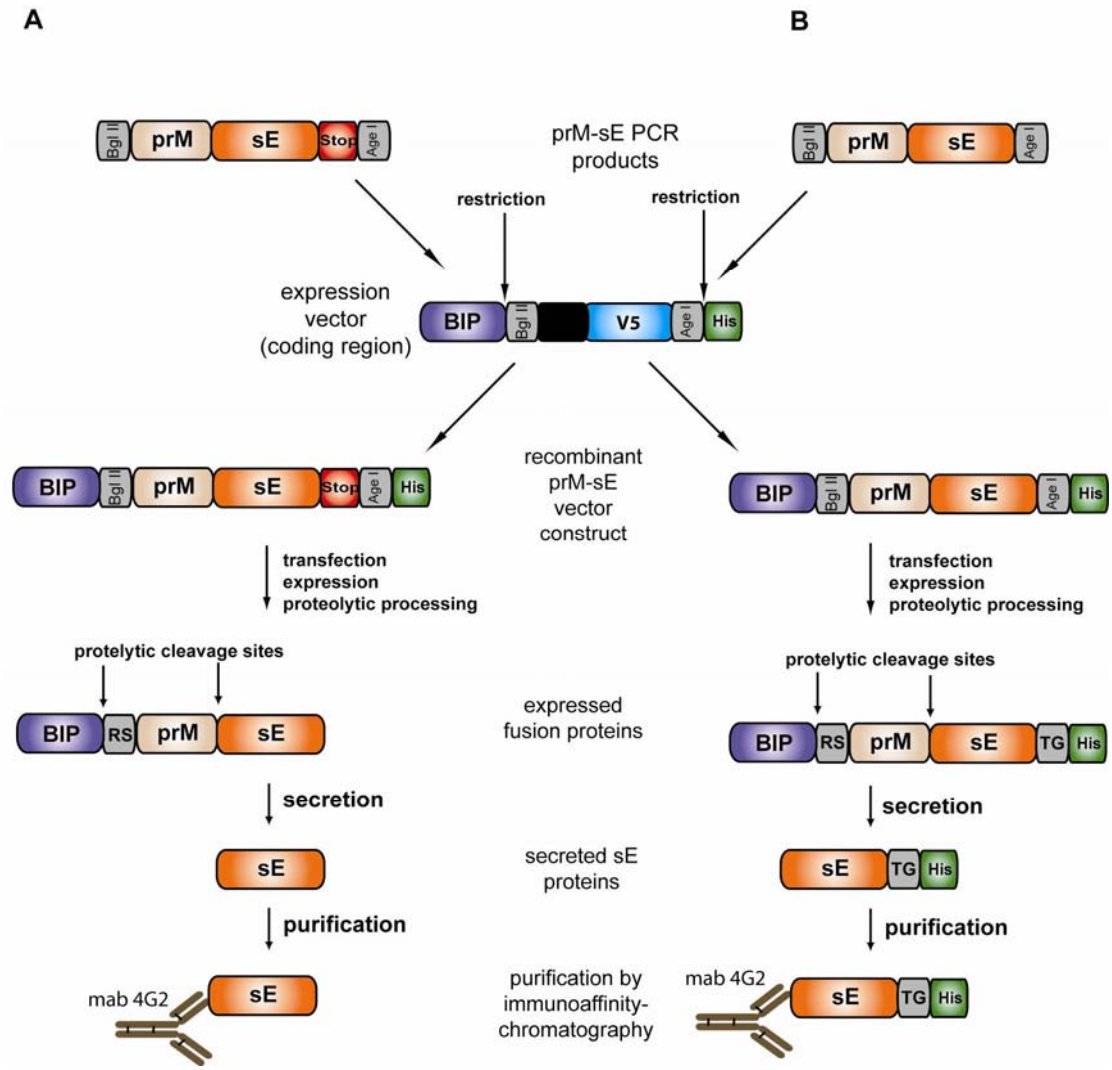

Fig. 4.6 Strategy for the cloning, expression, and purification of recombinant sE proteins
(A) Cloning, expression, and purification of sE proteins used as immunogens in mice
(B) Cloning, expression, and purification of sE-His proteins used as antigens in immunological assays. Soluble E (sE) is colored in orange, prM in pink, the BIP signal sequence in purple, the V5 epitope in blue, stop-codons (Stop) in red, His-tags in green, restriction sites and the amino acids (TG=threonine, glycine; RS=arginine, serine) they encode in grey.

4.2.1.2.2 Generation of stably transfected cell lines

To generate cell lines for the continuous expression of recombinant protein, the recombinant expression plasmids were co-transfected together with a blasticidin resistance-encoding plasmid into Schneider 2 (S2) Drosophila cells. Blasticidin selection was applied for two weeks to obtain stably transfected cells. Expression was induced by the addition of Cu^{2+}. To test for successful secretion of recombinant protein into the medium, aliquots of the cell culture supernatant from induced cells were analyzed by E protein-specific quantitative ELISA (data not shown).

4.2.1.2.3 Expression and purification of the recombinant sE proteins

For the expression of recombinant sE proteins, stably transfected cells were induced by the addition of Cu^{2+} to the culture medium and the cell culture supernatant was harvested by centrifugation 7 to 11 days post induction. The recombinant sE proteins were purified from the cell culture supernantant by immunoaffinity chromatography using the purified broadly flavivirus cross-reactive monoclonal antibody 4G2. As determined by SDS-ELISA, this procedure allowed the recovery of 60 to 70% of the sE from the starting material. To asses the purity, the eluted and buffer-exchanged proteins were subjected to SDS-PAGE and densitometric analysis. As shown in Fig. 4.7, the recombinant proteins had a purity of at least 90%.

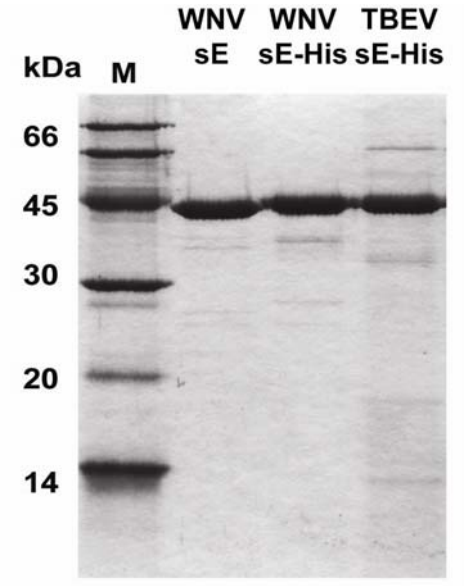

Fig. 4.7 SDS-PAGE and Coomassie staining of purified recombinant sE proteins
The lanes of the WN sE, sE-His and TBE sE-His proteins are indicated. M: molecular weight markers.

4.2.1.2.4 Assessment of the oligomeric structure of the recombinant sE proteins

To analyze the oligomeric state of the purified sE proteins, a chemical cross-linking experiment was performed using dimethylsuberimidate (DMS) and sE dimers obtained from infectious TBE virions (vTBEV sE) as controls (79). As shown in Fig.4.8, both recombinant TBE virus sE-His and the dimer control (vTBEV sE) showed two bands corresponding to an E dimer confirming the dimeric nature of the recombinant TBE virus sE protein. In contrast, WNV sE and sE-His migrated as monomers. Only a faint band at the molecular weight of a dimer was present indicating that either the recombinant sEs of WN virus are predominantly monomers or that they cannot be cross-linked with DMS.

For further investigation of their oligomeric state, the recombinant sE proteins were subjected to sucrose density gradient analysis at alkaline (pH 8.0) and acidic pH (pH 6.0). In general, E protein dimers are stable at pH 8.0, but dissociate into monomers upon acidification, both having different migration behaviours in sucrose density gradients. The recombinant sE proteins and, as a control, the sE dimers from infectious TBE virions (vTBEV sE), were pre-incubated at pH 6.0 or pH 8.0, applied onto sucrose density gradients with respective pH and subjected to ultra-

centrifugation. After centrifugation the gradient was fractionated and the distribution of the sE proteins in the fractions was analyzed by quantitative E protein-specific SDS-ELISA (Fig. 4.9). As expected, TBE sE from infectious virions sedimented as dimers at pH 8.0 and monomers at pH 6.0 (Fig. 4.9A, B, C). Recombinant TBE virus sE-His (Fig. 4.9A) showed the same sedimentation behaviour as the viral sE dimer, confirming the result of the chemical cross-linking experiment. The WN virus sE proteins (Fig. 4.9 B, C) in contrast, sedimented as monomers both at pH 6.0 and pH 8.0, again consistent with the cross-linking experiment.

These data indicate that the recombinant TBE virus sE-His protein was a dimer, whereas the sE and sE-His of WN virus formed predominantly monomers.

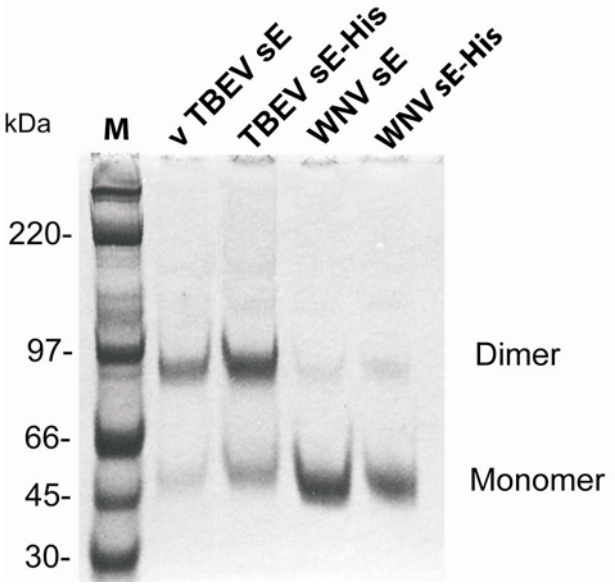

Fig. 4.8 SDS-PAGE and Coomassie staining of DMS-treated recombinant sE proteins.
The lanes of the vTBEV sE (control) and the recombinant sEs, the molecular weight markers (M) and the positions of the monomer and dimer are indicated.

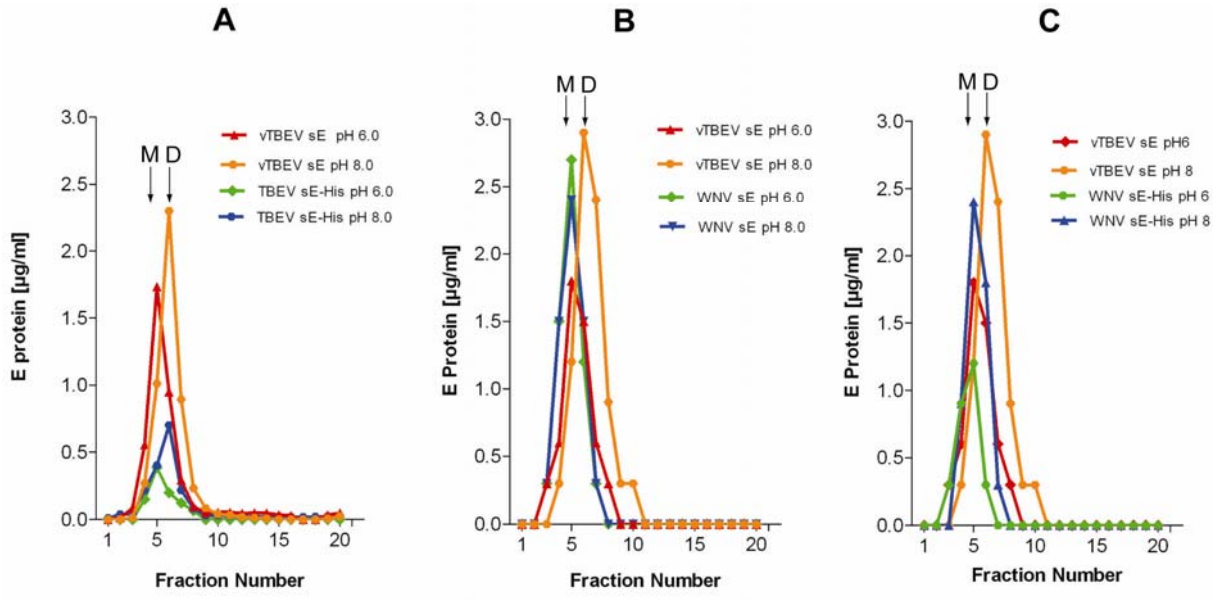

Fig. 4.9 Sedimentation analyses of recombinant sE proteins.

Distribution of the recombinant sE proteins (indicated by the colour code in the inset) in the sucrose density gradient (pH 8.0 and 6.0) as determined by SDS-ELISA. In all panels, the sE isolated from infectious TBE virions (vTBE sE) was used as a control. Positions of the monomer (M) and dimer (D) are indicated by an arrow.

(A) Recombinant TBEV sE-His

(B) Recombinant WNV sE

(C) Recombinant WNV sE-His

4.2.1.2.5 Evidence for proper folding of purified recombinant sE proteins

To analyze proper folding, recombinant sEs were subjected to an analysis with conformation sensitive mabs using Western blotting under non-reducing conditions. For this assay, non-treated and S-alkylated sEs were incubated with polyclonal control sera, DIII-specific and broadly cross-reactive conformation sensitive mabs, which only react when the disulfide bridges are properly formed. Fig. 4.10 shows Western blots with a representative set of mabs. The S-alkylated and untreated sEs reacted with the polyclonal control sera (TBE virus-specific, KVIII; WN virus-specific, KIS2), but the monoclonal antibodies (TBE virus specific A1 (DII), B3, B4 (DIII), (32); WN virus specific E16, E24 (DIII), (70)) reacted only with the non-treated but not with the S-alkylated forms. This is an indication for the proper formation of the disulfide bridges suggesting that the sE proteins had a correct conformation.

To compare the overall fold of the recombinant TBE virus sE-His with that of the sE isolated from infectious TBE virions (vTBEV sE), a blocking ELISA with conformation-dependent monoclonal antibodies was carried out. Figure 4.11 shows for DI (IC3), DII (A3) and DIII (B4) -specific monoclonal antibodies that the blocking pattern is identical for recombinant and viral sE, which is consistent with proper folding of the recombinant sE protein.

To summarize, the chosen cloning, expression and purification strategy led to the successful production of high amounts of at least 90% pure recombinant sE proteins in a correct conformation.

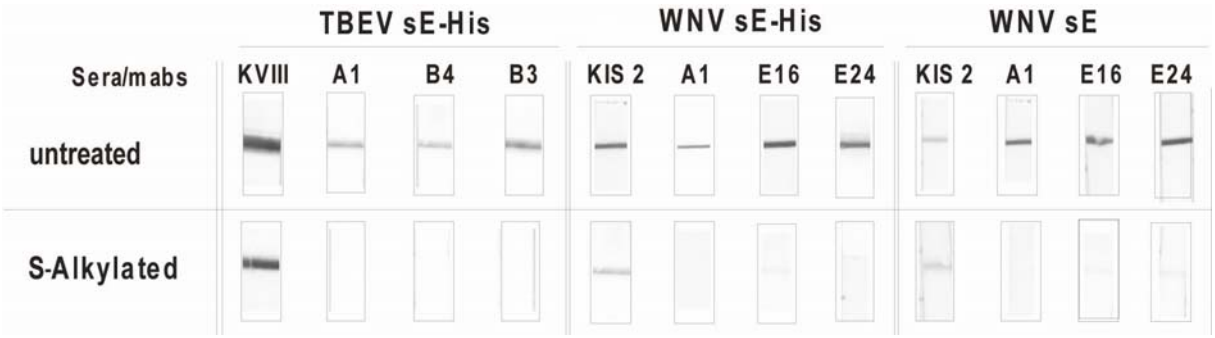

Fig. 4.10 Western blot analyses of untreated and S-alkylated purified sEs.

The sEs were subjected to SDS-PAGE and Western blotting under non-reducing conditions. Immunodetection was carried out with polyclonal TBE (KVIII) and WN (KIS2) virus-specific rabbit sera and the conformation-dependent mabs A1 (broadly cross-reactive), B3, B4 (TBE virus DIII-specific) and E16, E24 (WN virus DIII-specific) (70).

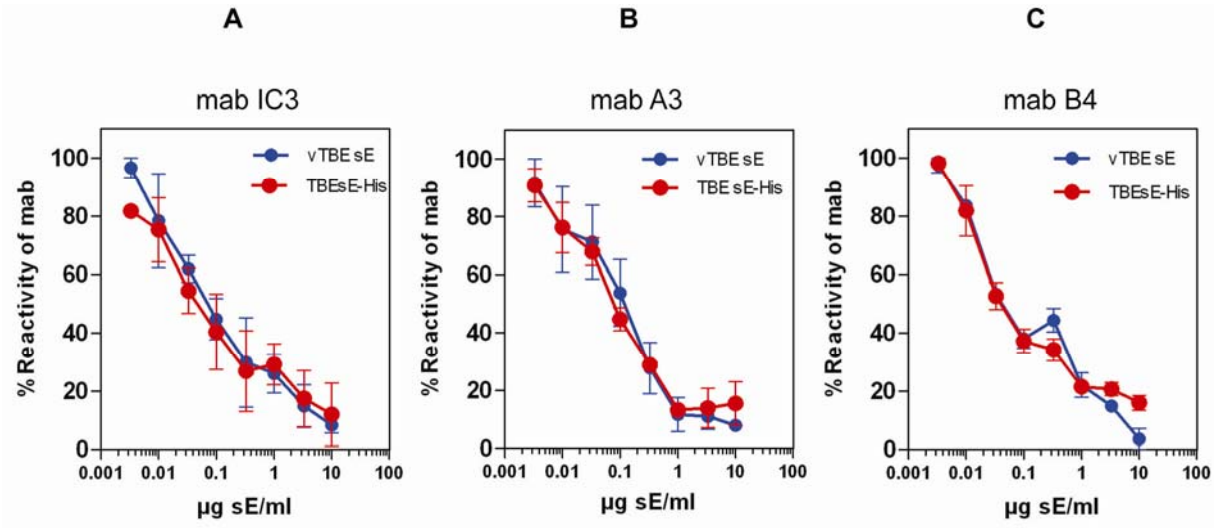

Fig. 4.11 Blocking ELISA with mabs, recombinant TBE sE-His and TBE sE isolated from infectious virions.

Titration curves of mabs IC3 (DI-specific), A3 (DII-specific), and B4 (DIII-specific) in TBE virion ELISA after pre-incubation with recombinant TBE sE-His (red curves) and TBE sE obtained from infectious virions (79) (vTBE sE, blue curves). The error bars represent the standard error of the means obtained in three independent determinations.

4.2.2 Dose-finding immunisation experiment in mice

It was the primary goal of this thesis to assess the induction of neutralizing antibodies by different WN virus antigens in different prime boost regimens. Since the specific immunogenicity of different physical forms of the antigen can be different (37), a dose finding experiment in mice had to be conducted.

The conditions for the induction of a neutralizing antibody response using whole inactivated virus as an immunogen in mice was already established by previous studies conducted in our group (data not shown). Therefore, the dose finding experiment was performed with purified DIII and sE. At this stage of the project, recombinant WN virus antigens were not yet available. For this reason, the already purified recombinant TBE virus DIII and the sE isolated from infectious TBE virions (79) were used as analogues for mouse immunizations. Groups of 6-8 week old female C57/BL6 mice (4 mice per group) were immunized three times at an interval of two weeks with 1, 5 or 20µg of DIII and sE, with and without aluminium hydroxide ($Al(OH)_3$), subcutaneously into the skin at the neck. Blood samples were taken before each of the immunizations and 14 days after the third and the sera of each group were pooled. The serum pools were analyzed by TBE virus specific IgM and IgG ELISAs and the functional activity was determined by TBE virus neutralization assays.

4.2.2.1 Analyses of DIII post-immunization sera

Groups which received recombinant DIII without aluminium hydroxide did not show any reactivity in the TBE virus specific IgM and IgG ELISA indicating that soluble DIII alone was not sufficiently immunogenic under the conditions used (data not shown). In contrast, DIII adsorbed to aluminium hydroxide induced a TBE virus specific IgM and IgG antibody response (Fig. 4.12A and B, respectively). IgM titers in groups receiving 1µg of DIII were below the detection limit even after the third immunization (Fig. 4.12A). The induction of IgG antibodies was clearly dose-dependent (Fig. 4.12B) and neutralizing antibody titers (NT_{50}) were only detectable after immunization with two or three doses of 20µg (Fig. 4.12C).

4.2.2.2 Analyses of sE post-immunization sera

sE obtained from infectious virus induced a specific IgM and IgG antibody response with and without the use of aluminium hydroxide as an adjuvant (Fig 4.13 A-D). IgM (Fig. 4.13A, B), IgG (Fig. 4.13C, D) and neutralizing antibodies (NT_{50}) (Fig. 4.13E, F) were detected in all samples at least after two doses. In the group receiving sE+aluminium hydroxide, IgM antibodies were measured already after a single dose (Fig. 4.13B) in contrast to mice receiving sE only, which were tested positive for IgM after the second dose (Fig. 4.13A). Highest IgG titers were detected in groups receiving three doses and appeared to be slightly higher in groups receiving adjuvant (Fig. 4.13D). As shown in Fig. 4.13 E and F, neutralizing antibodies (NT_{50}) were induced by all doses (+/- aluminium hydroxide), at least after two immunizations.

4.2.2.3 Conclusions

The dose-finding experiment provided an excellent basis for the selection of the amounts of antigens to be used in sequential and prime-boost immunization studies. DIII was the weakest immunogen and required a dose of 20µg (+ aluminium hydroxide), followed by sE (5µg) and inactivated virion (1µg), as determined in previous analyses.

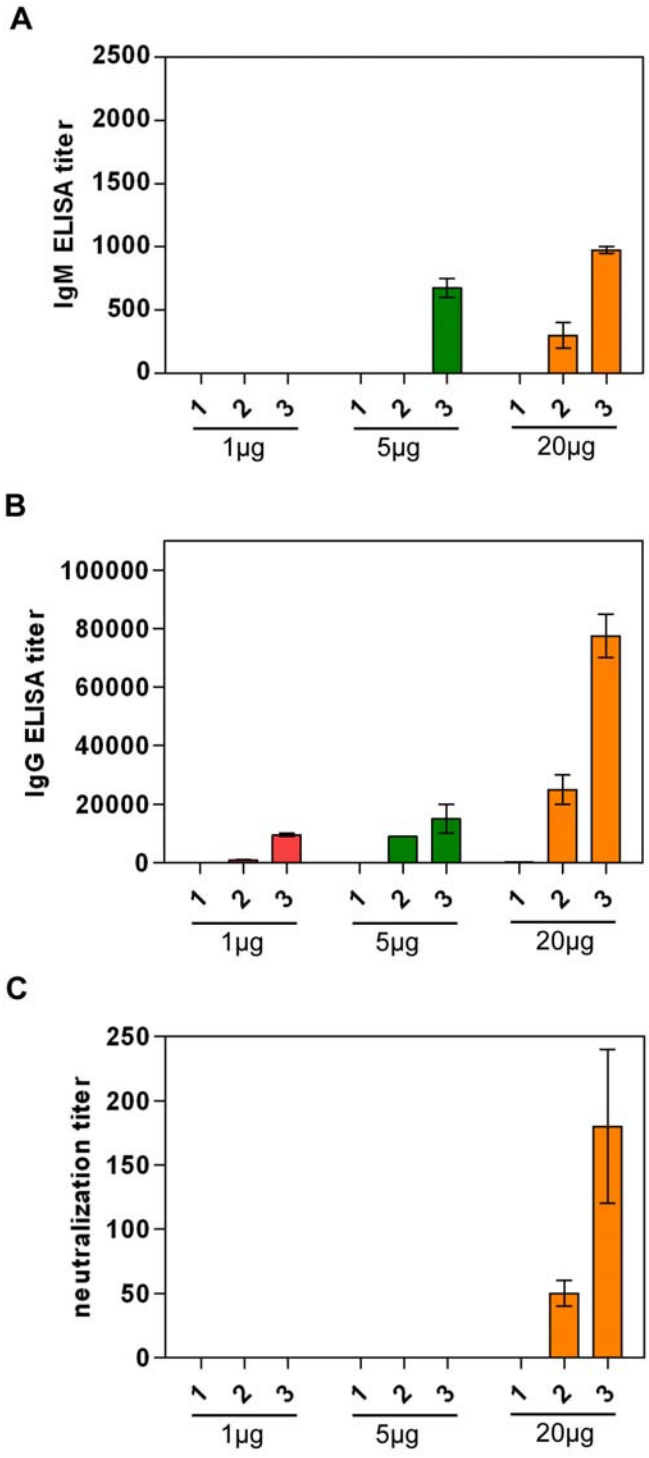

Fig. 4.12 Analyses of TBE virus DIII post-immunization sera by TBE virus specific ELISAs and neutralization assays.

(A) TBE virus-specific IgM ELISA titers.

(B) TBE virus-specific IgG ELISA titers.

(C) TBE virus-specific neutralization titers (NT_{50}).

In all panels, the dose of the immunogens in µg and the number of immunizations are indicated on the x-axis. Serum pools from mice receiving 1, 5, or 20µg are indicated in red, green and orange bars, respectively. The error bars represent the standard errors of the means obtained in two independent determinations.

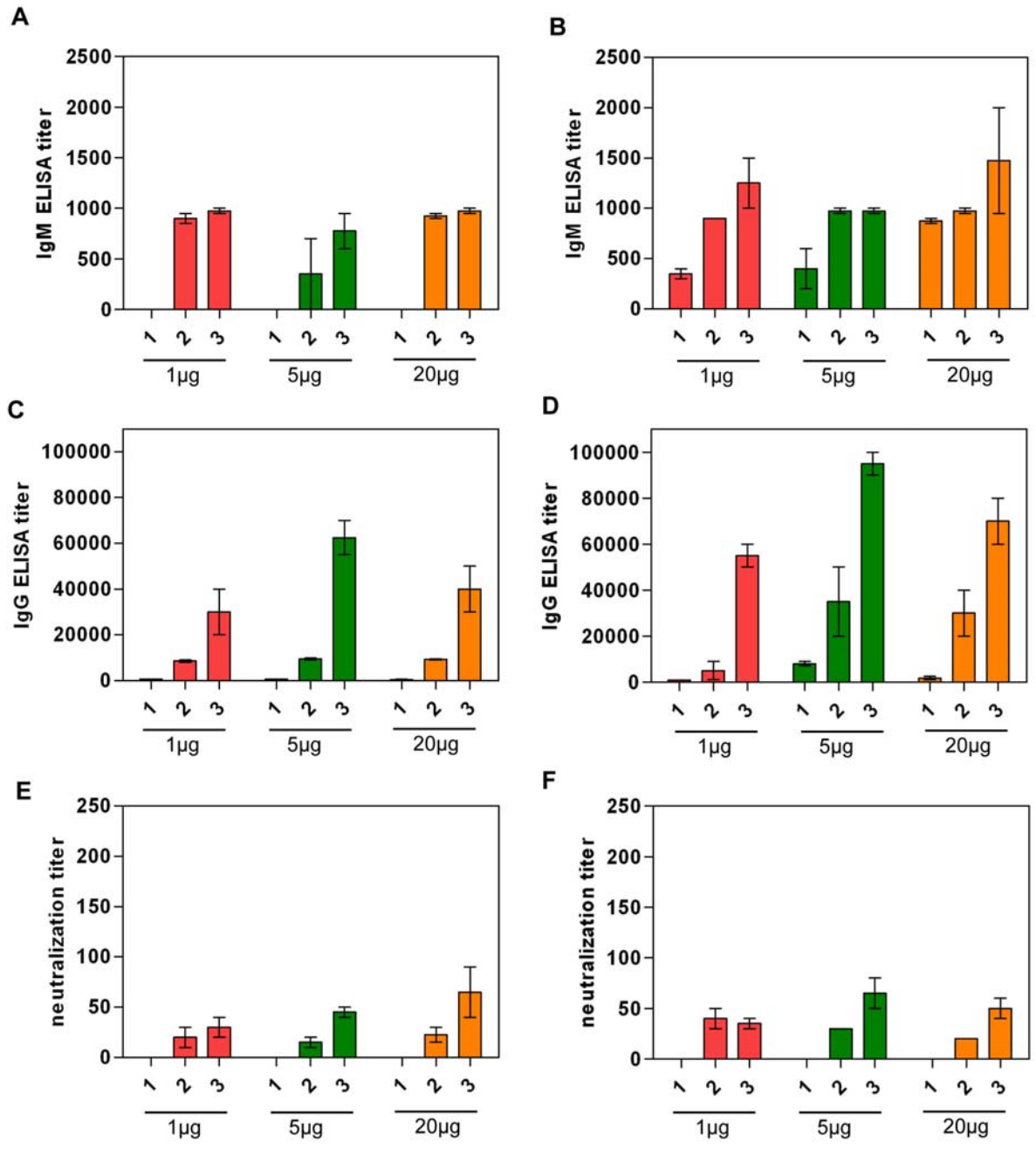

Fig. 4.13 Analyses of TBE virus sE post-immunization sera by TBE virus specific ELISAs and neutralization assays.

(A, B) TBE virus-specific IgM ELISA titers in sE (A) and sE+Al(OH)$_3$ (B) post-immunization sera.

(C, D) TBE virus-specific IgG ELISA titers in sE (C) and sE+Al(OH)$_3$ (D) post-immunization sera.

(E, F) TBE virus-specific neutralization titers (NT_{50}) in sE (E) and sE+Al(OH)$_3$ (F) post-immunization sera.

In all panels, the dose of the immunogens in µg and the number of immunizations are indicated on the x-axis. Serum pools from mice receiving 1, 5, and 20µg are indicated by red green and orange bars, respectively. The error bars represent the standard errors of the means obtained in two independent determinations.

4.2.3 Development of immunological assays

The goal of this part of the thesis was to establish assay formats that allowed the analysis of the fine-specificity of the polyclonal antibody response in mice after immunization with WN virus antigens (inactivated virion, recombinant sE and DIII). To determine serum antibody titers, ELISAs employing different antigens were established. To quantify the contribution of a specific subset of antibodies to the neutralizing antibody response, serum depletion assays were developed.

4.2.3.1 Development of a DIII ELISA format

For the determination of DIII-specific IgG antibody titers, purified recombinant DIII-His proteins of WN and TBE viruses were used as antigens in ELISA. For the establishment of this assay, TBE DIII-His and the TBE DIII-specific mab B4 were used to compare three different ELISA formats:

1) DIII coated directly to the plate (Fig. 4.15A).
2) Catching of DIII via the His-tag on Ni^{2+} coated plates (Fig. 4.15B).
3) Catching of DIII via the His-tag by anti-His-tag antibody coated plates (Fig. 4.15C).

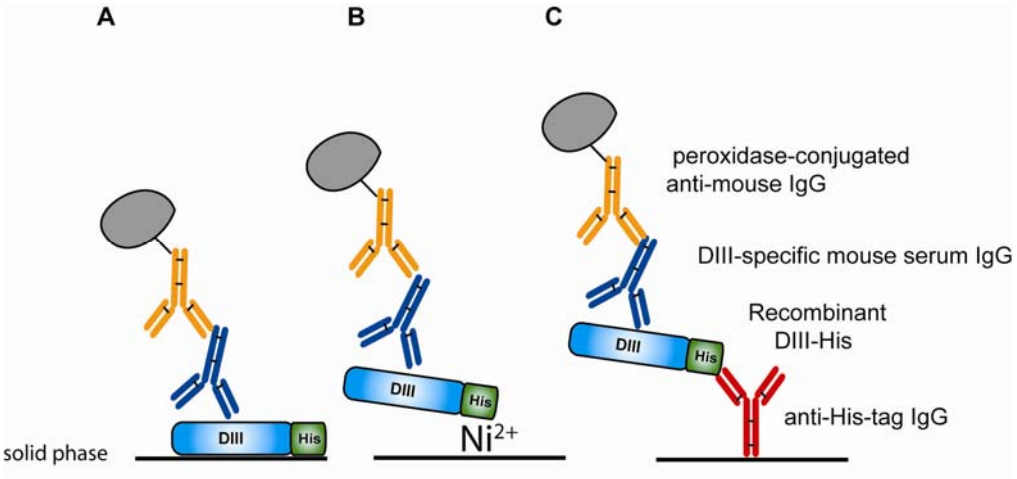

Fig. 4.15 Schematic representations of different DIII ELISA formats.
(A) ELISA format with DIII coated directly to the solid phase of the microtiter plate.
(B) ELISA format using catching of DIII-His by Ni^{2+}.
(C) ELISA format using anti-His-tag antibody for catching of DIII-His.

As shown in Fig. 4.16, the highest specific reactivities were obtained in the format using the anti-His tag mab for catching, followed by direct coating of DIII to the solid phase and Ni^{2+} catching, which was least effective. Therefore, the catching ELISA format using the anti-His-tag antibody was further optimized. To investigate the specificity of this assay, a WN virus DIII-specific mouse post-immunization serum was titrated on recombinant WN virus DIII-His and on plates without antigen. To test for possible background reactivities, sera from naïve mice were included in the analysis. To compare the sensitivity of the DIII ELISA to that of the standard virion ELISA using inactivated whole WN virus as an antigen, the post-immunization serum was analyzed in both assays in parallel. As shown in Fig. 4.17, the DIII serum showed almost no unspecific reactivity on plates without DIII but strong reactivity with DIII and virus, reaching higher titers in the DIII-ELISA format. Background reactivity was minimal, as assessed by the use of the negative mouse sera (data not shown). This demonstrates the successful establishment of a highly sensitive ELISA for the determination of DIII-specific antibody titers.

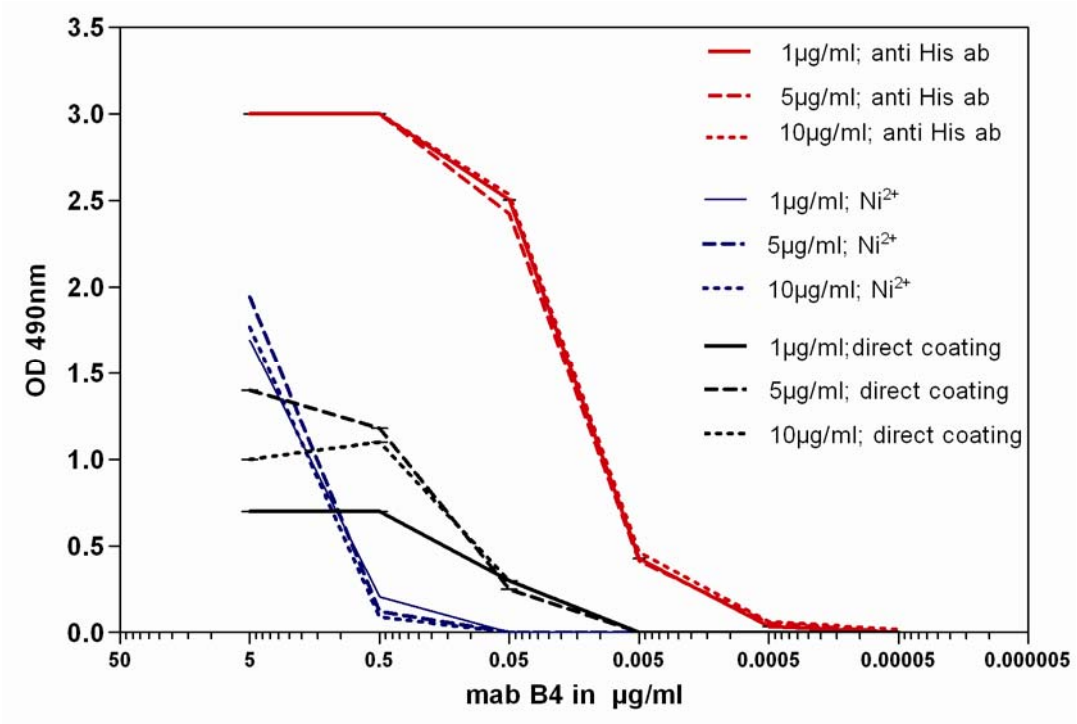

Fig. 4.16 Comparison of the reactivity of a DIII-specific mab in three different DIII ELISA formats.

Titration curves of TBE virus DIII specific mab B4 obtained in ELISA with 1, 5 and 10µg/ml recombinant TBE virus DIII-His directly coated to the plate (black lines), caught by Ni^{2+} (blue lines) or anti-His-tag antibody (red lines).

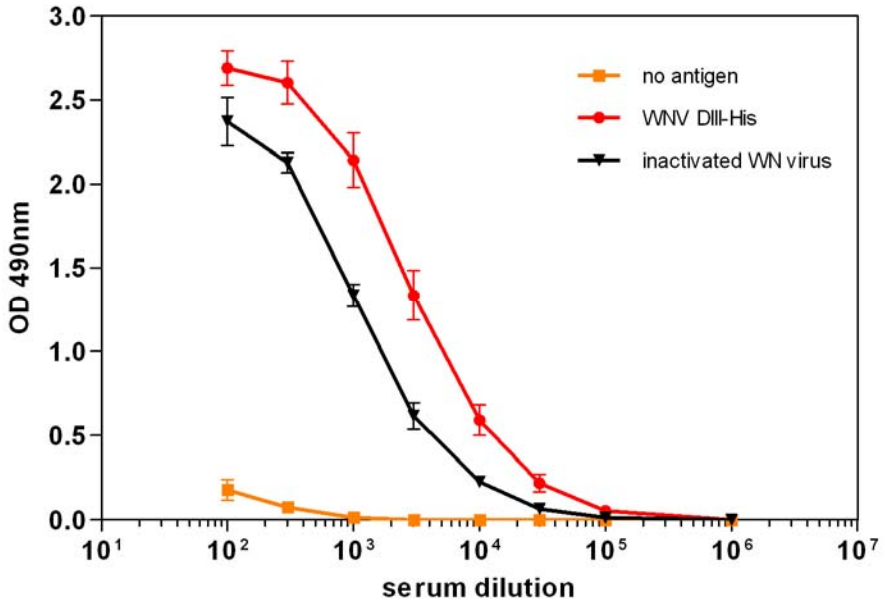

Fig. 4.17 Reactivity of a DIII specific mouse post-immunization serum in DIII and virion ELISAs. Titration curves of a WN virus DIII post-immunization serum on plates without antigen, WN virus DIII-His or whole inactivated WN virion, indicated in orange, red and black respectively. The error bars represent the standard error of the means obtained in three independent determinations.

4.2.3.2 Development of an sE ELISA format

For the determination of E protein-specific IgG antibody titers in TBE and WN virus sE-specific mouse post-immunization sera, purified recombinant sE-His proteins of WN and TBE virus were used as antigens in a catching ELISA format using the anti-His-tag antibody (compare Fig. 4.15C). Background reactivities were assessed by the use of sera from naïve mice. Comparison was also made with the standard virion ELISAs using the respective formalin-inactivated whole virus as antigen.

The sE ELISA formats exhibited almost no unspecific background reactivity as assessed by the use of sera from naïve mice (data not shown). As shown in Fig. 4.18A for the WN and in 4.18B for the TBE virus sE serum, comparable titers were reached in the sE and virion ELISAs, but the maximum absorbance was higher in the virion ELISA.

To investigate the suitability of the TBE virus sE ELISA for the detection of broadly flavivirus cross-reactive antibodies, an experiment using the cross-reactive mabs 4G2 and A1 was carried out in comparison to the virion ELISA. The experiment revealed that with both mAbs, higher specific reactivities were reached in the sE

compared to the virion ELISA (data not shown), suggesting a higher sensitivity of the sE ELISA for the detection of cross-reactive abs. As a further analysis of cross-reactivity, a WN virus-specific mouse post-immunization serum was titrated in the TBE virus sE and virion ELISAs. Figure 4.18B shows that - also with the polyclonal serum - higher titers were reached in the sE ELISA, confirming the results obtained with the cross-reactive mabs.

These results demonstrate the successful establishment of a sensitive ELISA format for the determination of homologous sE-reactive and broadly cross-reactive antibody titers.

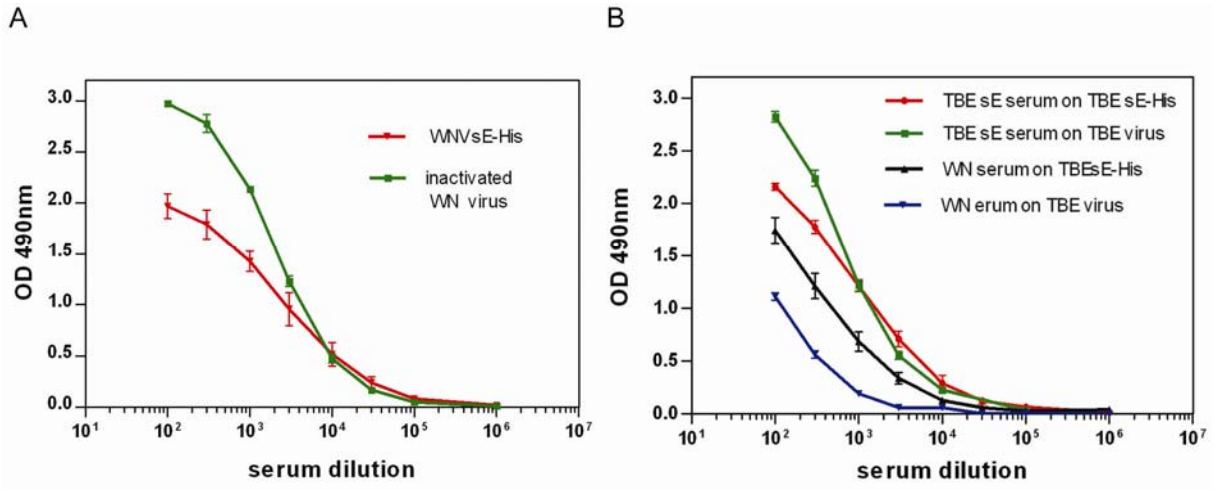

Fig. 4.18 Titration curves of sE post-immunization sera in sE and virion ELISAs.
(A) Titration curves of WN virus sE serum in WN sE (red curve) and WN virion ELISA (green curve). (B) Titration curves of TBE virus specific sE post-immunization serum in a TBE virus sE (red curve) and virion ELISA (green curve) and of a WN virus-specific serum in a TBE sE (black curve) and TBE virion ELISA (blue curve). The error bars represent the standard error of the means obtained in three independent determinations.

4.2.3.3 Development of antibody depletion assays

To determine the contribution of DIII-specific and broadly cross-reactive antibodies to the total neutralizing antibody response in polyclonal mouse post-immunization sera, antibody depletion assays were developed, using recombinant WN virus DIII-His, and heterologous TBE virus sE-His, respectively. The principle of the depletion assay is shown in Fig. 4.19. The recombinant His-tagged proteins were bound to

Co^{2+} coated magnetic beads via their His-tags and the loaded beads were subsequently incubated with the mouse post-immunization sera. Finally, the beads together with the bound antigen-antibody complexes were removed by applying magnetic force, leaving the depleted serum in the supernatant, which was further analyzed by ELISA and virus neutralization assays.

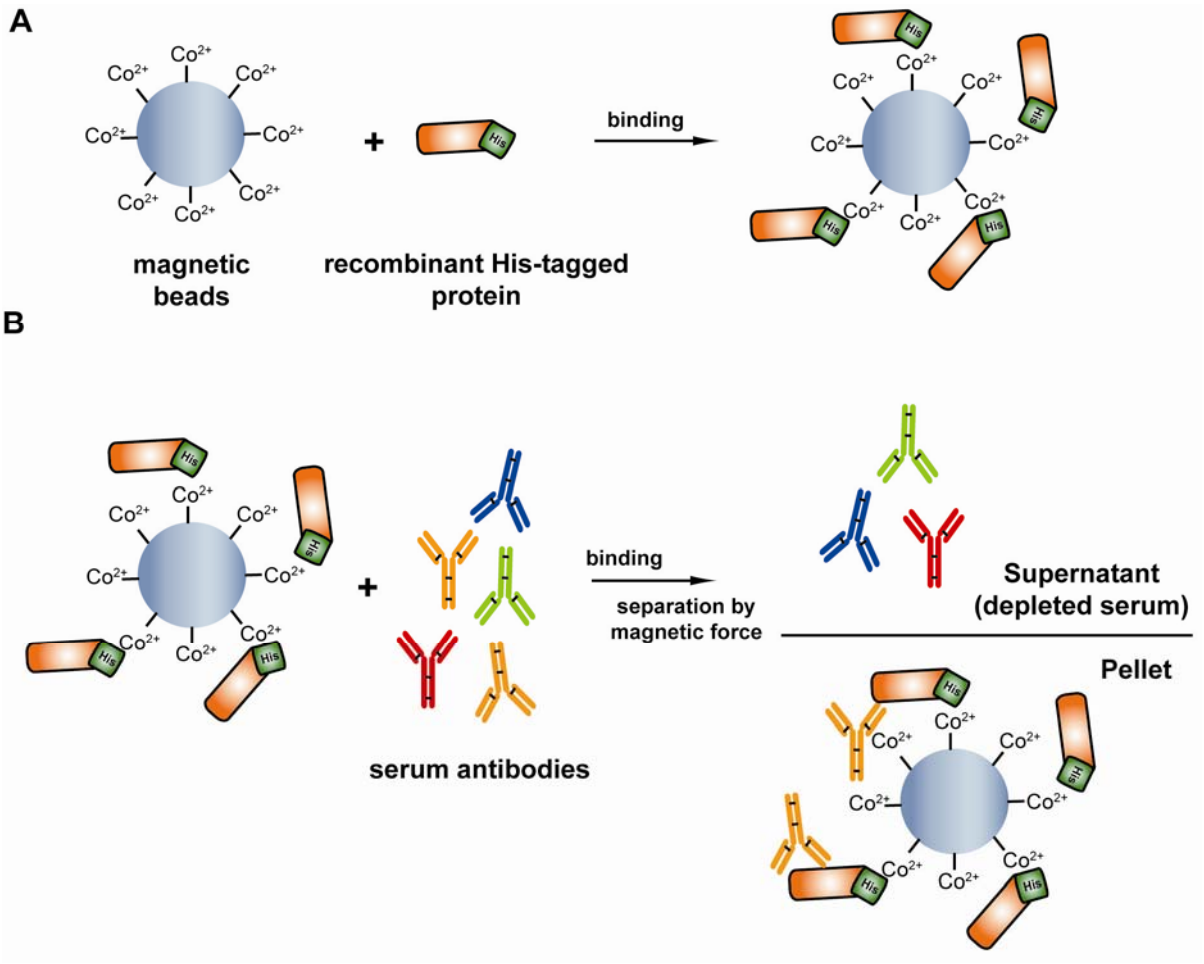

Fig. 4.19 Schematic representation of the antibody depletion assay.

(A) Binding of the His-tagged recombinant protein to the beads.

(B) Serum depletion with antigen-loaded beads.

4.2.3.4 Depletion of DIII-reactive antibodies

For establishing the depletion of DIII-specific antibodies from sera, Co^{2+} coated magnetic beads were incubated with 1, 5 and 10µg of DIII-His. After removal of the beads by magnetic force, analysis of the supernatant by SDS-PAGE and

densitometric analysis showed that about 40% of the DIII-His used for the binding reaction bound to the beads (data not shown).

For the establishment of depletion of DIII-reactive antibodies from polyclonal mouse sera, a WN virus DIII-specific mouse post-immunization serum was incubated with WN virus DIII-His loaded beads (1µg of recombinant WN virus DIII-His was used for binding to the beads). The supernatant, representing the depleted serum, was separated from the beads by magnetic force and further analyzed. To determine the efficiency of the serum depletion, the DIII-reactive antibody titers before and after depletion were determined by WN virus DIII-His specific ELISA. The ELISA revealed that a depletion efficiency of 98% was reached after performing three consecutive depletion reactions (Fig. 4.20A). To test whether unspecific binding of antibodies to the beads occurred, a WN virus DIII-specific mouse post-immunization serum was incubated with blank beads, beads coated with TBE and WN virus DIII-His. The depleted sera and the serum before depletion were analyzed by WN virus DIII-specific ELISA. As shown in Fig. 4.21A, only the WN virus DIII-His coated beads were able to remove the WN DIII-reactive antibodies quantitatively. The titration curves of sera treated with blank beads and beads coated with TBE virus DIII-His were almost identical to the non-depleted control, indicating that no unspecific adsorption occurred.

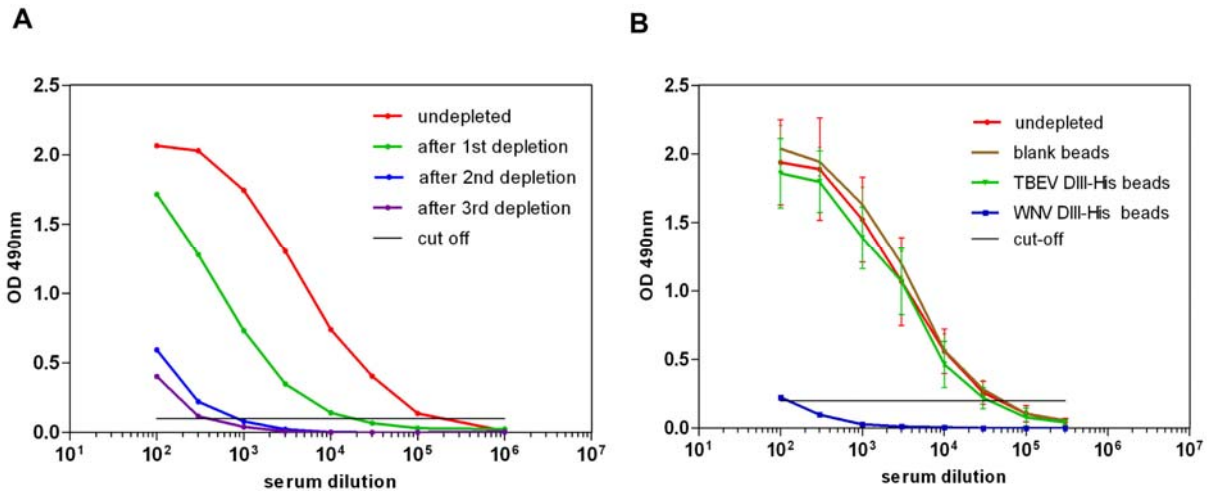

Figure 4.20 Analyses of a DIII-specific post-immunization serum after depletion with DIII-His by DIII-specific ELISAs.

(A) Aliquots of the supernatants after each depletion reaction with WN virus DIII-His loaded beads were analyzed by DIII-specific ELISA. Titration curves of the depleted sera after the first, second, and third round of depletion are indicated by green, blue, and purple lines, respectively.

(B) Analysis of a WN virus DIII post-immunization serum before (red curve) and after depletion using blank beads (red curve), TBE virus DIII-His (green curve) and WN virus DIII-His loaded beads (blue curve) by WN virus DIII-His specific ELISA.
The cut-offs, indicated by a black horizontal line, were deduced from the reactivity of sera from naïve mice. The error bars in panel B represent the standard error of the means obtained in three independent determinations.

4.2.3.5 Depletion of TBE virus sE-reactive antibodies from TBE and WN virus-specific sera

For establishing the depletion of TBE virus sE-reactive antibodies from sera, 1, 5, 10 µg of TBE virus sE-His were incubated with magnetic beads, which were then separated from the supernatant by magnetic force. After removal of the beads, the supernatant was subjected to analysis by SDS-ELISA to determine the binding efficiency. The TBE virus sE-His bound almost quantitatively (>90%) to the beads (data not shown). For the depletion of TBE sE reactive antibodies, a TBE virus sE-specific mouse post-immunization serum was incubated with the loaded beads. To assess the depletion efficiency the depleted serum was analyzed by TBE virus sE-His-specific ELISA. The ELISA revealed that – after performing three consecutive depletion reactions – more than 95% of the TBE virus sE-reactive antibodies were removed (data not shown). To test whether the established conditions are suitable for efficient depletion of broadly cross-reactive antibodies, a WN virus-specific mouse post-immunization serum and, as a control, the TBE virus sE-specific serum were incubated with TBE sE-His coated beads. The depletion efficiency was then assessed by TBE virus sE-His specific ELISA. As shown in Fig. 4.21 the reactivity was strongly reduced after depletion, showing a reduction in reactivity of more than 90%.

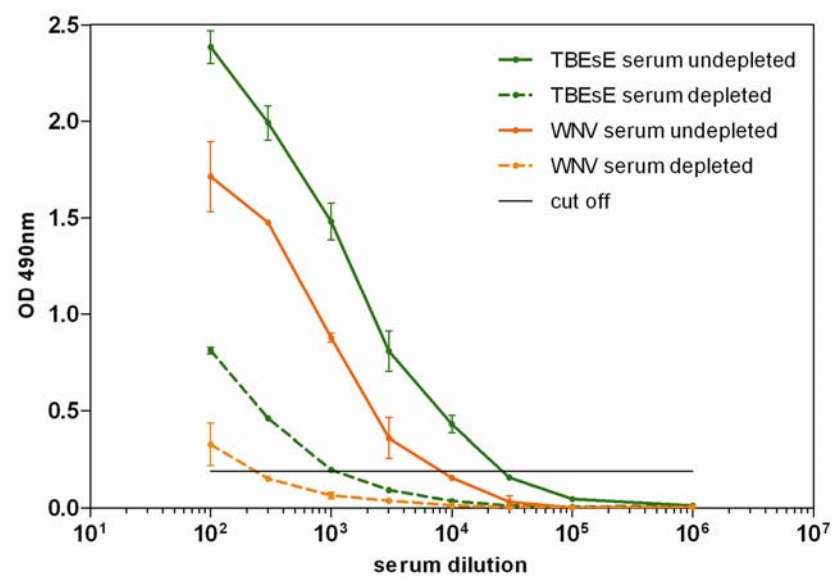

Figure 4.21 Analysis of WN and TBE virus sE post-immunization sera after depletion with TBE sE-His by TBE sE-His-specific ELISA

Analysis of a TBE virus sE (green curves) and a WN virus-specific post-immunization serum, (orange curves) before (solid lines) and after depletion (dashed lines) with TBE sE-His, by TBE sE-His-specific ELISA. The error bars represent the standard errors of the means obtained in three independent determinations.

5. References

1. **Alka, K. Bharati, Y. P. Malik, and S. Vrati.** 2007. Immunogenicity and protective efficacy of the E. coli-expressed domain III of Japanese encephalitis virus envelope protein in mice. Med Microbiol Immunol **196:**227-31.
2. **Allison, S. L., K. Stadler, C. W. Mandl, C. Kunz, and F. X. Heinz.** 1995. Synthesis and secretion of recombinant tick-borne encephalitis virus protein E in soluble and particulate form. J Virol **69:**5816-20.
3. **Bakonyi, T., E. Ivanics, K. Erdelyi, K. Ursu, E. Ferenczi, H. Weissenbock, and N. Nowotny.** 2006. Lineage 1 and 2 strains of encephalitic West Nile virus, central Europe. Emerg Infect Dis **12:**618-23.
4. **Beasley, D. W., and A. D. Barrett.** 2002. Identification of neutralizing epitopes within structural domain III of the West Nile virus envelope protein. J Virol **76:**13097-100.
5. **Beltramello, M., K. L. Williams, C. P. Simmons, A. Macagno, L. Simonelli, N. T. Quyen, S. Sukupolvi-Petty, E. Navarro-Sanchez, P. R. Young, A. M. de Silva, F. A. Rey, L. Varani, S. S. Whitehead, M. S. Diamond, E. Harris, A. Lanzavecchia, and F. Sallusto.** The human immune response to Dengue virus is dominated by highly cross-reactive antibodies endowed with neutralizing and enhancing activity. Cell Host Microbe **8:**271-83.
6. **Bhardwaj, S., M. Holbrook, R. E. Shope, A. D. Barrett, and S. J. Watowich.** 2001. Biophysical characterization and vector-specific antagonist activity of domain III of the tick-borne flavivirus envelope protein. J Virol **75:**4002-7.
7. **Bonafe, N., J. A. Rininger, R. G. Chubet, H. G. Foellmer, S. Fader, J. F. Anderson, S. L. Bushmich, K. Anthony, M. Ledizet, E. Fikrig, R. A. Koski, and P. Kaplan.** 2009. A recombinant West Nile virus envelope protein vaccine candidate produced in Spodoptera frugiperda expresSF+ cells. Vaccine **27:**213-22.
8. **Calisher, C. H., N. Karabatsos, J. M. Dalrymple, R. E. Shope, J. S. Porterfield, E. G. Westaway, and W. E. Brandt.** 1989. Antigenic relationships between flaviviruses as determined by cross-neutralization tests with polyclonal antisera. J Gen Virol **70 (Pt 1):**37-43.

9. **Campbell, G. L., A. A. Marfin, R. S. Lanciotti, and D. J. Gubler.** 2002. West Nile virus. Lancet Infect Dis **2:**519-29.
10. **Chen, S., M. Yu, T. Jiang, Y. Deng, C. Qin, and E. Qin.** 2007. Induction of tetravalent protective immunity against four dengue serotypes by the tandem domain III of the envelope protein. DNA Cell Biol **26:**361-7.
11. **Cherrier, M. V., B. Kaufmann, G. E. Nybakken, S. M. Lok, J. T. Warren, B. R. Chen, C. A. Nelson, V. A. Kostyuchenko, H. A. Holdaway, P. R. Chipman, R. J. Kuhn, M. S. Diamond, M. G. Rossmann, and D. H. Fremont.** 2009. Structural basis for the preferential recognition of immature flaviviruses by a fusion-loop antibody. Embo J **28:**3269-76.
12. **Chu, J. H., C. C. Chiang, and M. L. Ng.** 2007. Immunization of flavivirus West Nile recombinant envelope domain III protein induced specific immune response and protection against West Nile virus infection. J Immunol **178:**2699-705.
13. **Chu, J. J., R. Rajamanonmani, J. Li, R. Bhuvanakantham, J. Lescar, and M. L. Ng.** 2005. Inhibition of West Nile virus entry by using a recombinant domain III from the envelope glycoprotein. J Gen Virol **86:**405-12.
14. **Clements, D. E., B. A. Coller, M. M. Lieberman, S. Ogata, G. Wang, K. E. Harada, J. R. Putnak, J. M. Ivy, M. McDonell, G. S. Bignami, I. D. Peters, J. Leung, C. Weeks-Levy, E. T. Nakano, and T. Humphreys.** Development of a recombinant tetravalent dengue virus vaccine: immunogenicity and efficacy studies in mice and monkeys. Vaccine **28:**2705-15.
15. **Crill, W. D., and G. J. Chang.** 2004. Localization and characterization of flavivirus envelope glycoprotein cross-reactive epitopes. J Virol **78:**13975-86.
16. **Crill, W. D., H. R. Hughes, M. J. Delorey, and G. J. Chang.** 2009. Humoral immune responses of dengue fever patients using epitope-specific serotype-2 virus-like particle antigens. PLoS One **4:**e4991.
17. **Crill, W. D., and J. T. Roehrig.** 2001. Monoclonal antibodies that bind to domain III of dengue virus E glycoprotein are the most efficient blockers of virus adsorption to Vero cells. J Virol **75:**7769-73.
18. **Dauphin, G., and S. Zientara.** 2007. West Nile virus: recent trends in diagnosis and vaccine development. Vaccine **25:**5563-76.
19. **Dejnirattisai, W., A. Jumnainsong, N. Onsirisakul, P. Fitton, S. Vasanawathana, W. Limpitikul, C. Puttikhunt, C. Edwards, T.**

Duangchinda, S. Supasa, K. Chawansuntati, P. Malasit, J. Mongkolsapaya, and G. Screaton. Cross-reacting antibodies enhance dengue virus infection in humans. Science **328:**745-8.

20. **Diamond, M. S., and R. S. Klein.** 2004. West Nile virus: crossing the blood-brain barrier. Nat Med **10:**1294-5.
21. **Diamond, M. S., T. C. Pierson, and D. H. Fremont.** 2008. The structural immunology of antibody protection against West Nile virus. Immunol Rev **225:**212-25.
22. **Dokland, T., M. Walsh, J. M. Mackenzie, A. A. Khromykh, K. H. Ee, and S. Wang.** 2004. West Nile virus core protein; tetramer structure and ribbon formation. Structure **12:**1157-63.
23. **Etemad, B., G. Batra, R. Raut, S. Dahiya, S. Khanam, S. Swaminathan, and N. Khanna.** 2008. An envelope domain III-based chimeric antigen produced in Pichia pastoris elicits neutralizing antibodies against all four dengue virus serotypes. Am J Trop Med Hyg **79:**353-63.
24. **Falconar, A. K.** 1999. Identification of an epitope on the dengue virus membrane (M) protein defined by cross-protective monoclonal antibodies: design of an improved epitope sequence based on common determinants present in both envelope (E and M) proteins. Arch Virol **144:**2313-30.
25. **Fauquet, C. M., M. A. Mayo, J. Maniloff, U. Desselberger, and L. A. Ball.** 2005. Virus Taxonomy. (San Diego: Elsevier Inc.).
26. **Ferlenghi, I., M. Clarke, T. Ruttan, S. L. Allison, J. Schalich, F. X. Heinz, S. C. Harrison, F. A. Rey, and S. D. Fuller.** 2001. Molecular organization of a recombinant subviral particle from tick-borne encephalitis virus. Mol Cell **7:**593-602.
27. **Francis, T., Jr., F. M. Davenport, and A. V. Hennessy.** 1953. A serological recapitulation of human infection with different strains of influenza virus. Trans Assoc Am Physicians **66:**231-9.
28. **Fritz, R., K. Stiasny, and F. X. Heinz.** 2008. Identification of specific histidines as pH sensors in flavivirus membrane fusion. J Cell Biol **183:**353-61.
29. **Giladi, M., E. Metzkor-Cotter, D. A. Martin, Y. Siegman-Igra, A. D. Korczyn, R. Rosso, S. A. Berger, G. L. Campbell, and R. S. Lanciotti.** 2001. West Nile encephalitis in Israel, 1999: the New York connection. Emerg Infect Dis **7:**659-61.

30. **Gromowski, G. D., N. D. Barrett, and A. D. Barrett.** 2008. Characterization of dengue virus complex-specific neutralizing epitopes on envelope protein domain III of dengue 2 virus. J Virol **82:**8828-37.

31. **Gubler, D. J.** 2007. The continuing spread of West Nile virus in the western hemisphere. Clin Infect Dis **45:**1039-46.

32. **Guirakhoo, F., F. X. Heinz, and C. Kunz.** 1989. Epitope model of tick-borne encephalitis virus envelope glycoprotein E: analysis of structural properties, role of carbohydrate side chain, and conformational changes occurring at acidic pH. Virology **169:**90-9.

33. **Guy, B., F. Guirakhoo, V. Barban, S. Higgs, T. P. Monath, and J. Lang.** Preclinical and clinical development of YFV 17D-based chimeric vaccines against dengue, West Nile and Japanese encephalitis viruses. Vaccine **28:**632-49.

34. **Guzman, M. G., L. Hermida, L. Bernardo, R. Ramirez, and G. Guillen.** 2010. Domain III of the envelope protein as a dengue vaccine target. Expert Rev Vaccines **9:**137-47.

35. **Gyure, K. A.** 2009. West Nile virus infections. J Neuropathol Exp Neurol **68:**1053-60.

36. **Halstead, S. B., S. Rojanasuphot, and N. Sangkawibha.** 1983. Original antigenic sin in dengue. Am J Trop Med Hyg **32:**154-6.

37. **Heinz, F. X., S. L. Allison, K. Stiasny, J. Schalich, H. Holzmann, C. W. Mandl, and C. Kunz.** 1995. Recombinant and virion-derived soluble and particulate immunogens for vaccination against tick-borne encephalitis. Vaccine **13:**1636-42.

38. **Heinz, F. X., R. Berger, W. Tuma, and C. Kunz.** 1983. A topological and functional model of epitopes on the structural glycoprotein of tick-borne encephalitis virus defined by monoclonal antibodies. Virology **126:**525-37.

39. **Heinz, F. X., and C. Kunz.** 1981. Homogeneity of the structural glycoprotein from European isolates of tick-borne encephalitis virus: comparison with other flaviviruses. J Gen Virol **57:**263-74.

40. **Hermida, L., R. Rodriguez, L. Lazo, R. Silva, A. Zulueta, G. Chinea, C. Lopez, M. G. Guzman, and G. Guillen.** 2004. A dengue-2 Envelope fragment inserted within the structure of the P64k meningococcal protein carrier enables

a functional immune response against the virus in mice. J Virol Methods **115:**41-9.

41. **Heyman, B.** 2000. Regulation of antibody responses via antibodies, complement, and Fc receptors. Annu Rev Immunol **18:**709-37.
42. **Hjelm, F., F. Carlsson, A. Getahun, and B. Heyman.** 2006. Antibody-mediated regulation of the immune response. Scand J Immunol **64:**177-84.
43. **Holzmann, H., M. Kundi, K. Stiasny, J. Clement, P. McKenna, C. Kunz, and F. X. Heinz.** 1996. Correlation between ELISA, hemagglutination inhibition, and neutralization tests after vaccination against tick-borne encephalitis. J Med Virol **48:**102-7.
44. **Huang, K. J., Y. C. Yang, Y. S. Lin, J. H. Huang, H. S. Liu, T. M. Yeh, S. H. Chen, C. C. Liu, and H. Y. Lei.** 2006. The dual-specific binding of dengue virus and target cells for the antibody-dependent enhancement of dengue virus infection. J Immunol **176:**2825-32.
45. **Kanai, R., K. Kar, K. Anthony, L. H. Gould, M. Ledizet, E. Fikrig, W. A. Marasco, R. A. Koski, and Y. Modis.** 2006. Crystal structure of west nile virus envelope glycoprotein reveals viral surface epitopes. J Virol **80:**11000-8.
46. **Kaufman, B. M., P. L. Summers, D. R. Dubois, W. H. Cohen, M. K. Gentry, R. L. Timchak, D. S. Burke, and K. H. Eckels.** 1989. Monoclonal antibodies for dengue virus prM glycoprotein protect mice against lethal dengue infection. Am J Trop Med Hyg **41:**576-80.
47. **Khanam, S., B. Etemad, N. Khanna, and S. Swaminathan.** 2006. Induction of neutralizing antibodies specific to dengue virus serotypes 2 and 4 by a bivalent antigen composed of linked envelope domains III of these two serotypes. Am J Trop Med Hyg **74:**266-77.
48. **Khanam, S., R. Pilankatta, N. Khanna, and S. Swaminathan.** 2009. An adenovirus type 5 (AdV5) vector encoding an envelope domain III-based tetravalent antigen elicits immune responses against all four dengue viruses in the presence of prior AdV5 immunity. Vaccine **27:**6011-21.
49. **Kiermayr, S., R. M. Kofler, C. W. Mandl, P. Messner, and F. X. Heinz.** 2004. Isolation of capsid protein dimers from the tick-borne encephalitis flavivirus and in vitro assembly of capsid-like particles. J Virol **78:**8078-84.
50. **Konishi, E., S. Pincus, E. Paoletti, R. E. Shope, T. Burrage, and P. W. Mason.** 1992. Mice immunized with a subviral particle containing the

Japanese encephalitis virus prM/M and E proteins are protected from lethal JEV infection. Virology **188:**714-20.

51. **Kuhn, R. J., W. Zhang, M. G. Rossmann, S. V. Pletnev, J. Corver, E. Lenches, C. T. Jones, S. Mukhopadhyay, P. R. Chipman, E. G. Strauss, T. S. Baker, and J. H. Strauss.** 2002. Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. Cell **108:**717-25.
52. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680-5.
53. **Lai, C. Y., W. Y. Tsai, S. R. Lin, C. L. Kao, H. P. Hu, C. C. King, H. C. Wu, G. J. Chang, and W. K. Wang.** 2008. Antibodies to envelope glycoprotein of dengue virus during the natural course of infection are predominantly cross-reactive and recognize epitopes containing highly conserved residues at the fusion loop of domain II. J Virol **82:**6631-43.
54. **Lanciotti, R. S., G. D. Ebel, V. Deubel, A. J. Kerst, S. Murri, R. Meyer, M. Bowen, N. McKinney, W. E. Morrill, M. B. Crabtree, L. D. Kramer, and J. T. Roehrig.** 2002. Complete genome sequences and phylogenetic analysis of West Nile virus strains isolated from the United States, Europe, and the Middle East. Virology **298:**96-105.
55. **Lanciotti, R. S., J. T. Roehrig, V. Deubel, J. Smith, M. Parker, K. Steele, B. Crise, K. E. Volpe, M. B. Crabtree, J. H. Scherret, R. A. Hall, J. S. MacKenzie, C. B. Cropp, B. Panigrahy, E. Ostlund, B. Schmitt, M. Malkinson, C. Banet, J. Weissman, N. Komar, H. M. Savage, W. Stone, T. McNamara, and D. J. Gubler.** 1999. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. Science **286:**2333-7.
56. **Ledizet, M., K. Kar, H. G. Foellmer, T. Wang, S. L. Bushmich, J. F. Anderson, E. Fikrig, and R. A. Koski.** 2005. A recombinant envelope protein vaccine against West Nile virus. Vaccine **23:**3915-24.
57. **Li, L., S. M. Lok, I. M. Yu, Y. Zhang, R. J. Kuhn, J. Chen, and M. G. Rossmann.** 2008. The flavivirus precursor membrane-envelope protein complex: structure and maturation. Science **319:**1830-4.
58. **Lieberman, M. M., D. E. Clements, S. Ogata, G. Wang, G. Corpuz, T. Wong, T. Martyak, L. Gilson, B. A. Coller, J. Leung, D. M. Watts, R. B. Tesh, M. Siirin, A. Travassos da Rosa, T. Humphreys, and C. Weeks-**

Levy. 2007. Preparation and immunogenic properties of a recombinant West Nile subunit vaccine. Vaccine **25:**414-23.

59. **Lieberman, M. M., V. R. Nerurkar, H. Luo, B. Cropp, R. Carrion, Jr., M. de la Garza, B. A. Coller, D. Clements, S. Ogata, T. Wong, T. Martyak, and C. Weeks-Levy.** 2009. Immunogenicity and protective efficacy of a recombinant subunit West Nile virus vaccine in rhesus monkeys. Clin Vaccine Immunol **16:**1332-7.
60. **Lindenbach, B. D., Thiel, H.-J., Rice, C.M.** 2007. Flaviviridae: the viruses and their replication, p. 1101-1152. *In* D. M. Knipe, Howley, P.M. (ed.), Fields Virology, 5th ed. Lippincott Williams & Wilkins Co., Philadelphia, PA., Philadelphia.
61. **Liu, W., H. Jiang, J. Zhou, X. Yang, Y. Tang, D. Fang, and L. Jiang.** Recombinant dengue virus-like particles from Pichia pastoris: efficient production and immunological properties. Virus Genes **40:**53-9.
62. **Lorenz, I. C., S. L. Allison, F. X. Heinz, and A. Helenius.** 2002. Folding and dimerization of tick-borne encephalitis virus envelope proteins prM and E in the endoplasmic reticulum. J Virol **76:**5480-91.
63. **Markoff, L.** 2003. 5'- and 3'-noncoding regions in flavivirus RNA. Adv Virus Res **59:**177-228.
64. **Modis, Y., S. Ogata, D. Clements, and S. C. Harrison.** 2003. A ligand-binding pocket in the dengue virus envelope glycoprotein. Proc Natl Acad Sci U S A **100:**6986-91.
65. **Modis, Y., S. Ogata, D. Clements, and S. C. Harrison.** 2005. Variable surface epitopes in the crystal structure of dengue virus type 3 envelope glycoprotein. J Virol **79:**1223-31.
66. **Mukhopadhyay, S., B. S. Kim, P. R. Chipman, M. G. Rossmann, and R. J. Kuhn.** 2003. Structure of West Nile virus. Science **302:**248.
67. **Nelson, S., C. A. Jost, Q. Xu, J. Ess, J. E. Martin, T. Oliphant, S. S. Whitehead, A. P. Durbin, B. S. Graham, M. S. Diamond, and T. C. Pierson.** 2008. Maturation of West Nile virus modulates sensitivity to antibody-mediated neutralization. PLoS Pathog **4:**e1000060.
68. **Nybakken, G. E., C. A. Nelson, B. R. Chen, M. S. Diamond, and D. H. Fremont.** 2006. Crystal structure of the West Nile virus envelope glycoprotein. J Virol **80:**11467-74.

69. **Nybakken, G. E., T. Oliphant, S. Johnson, S. Burke, M. S. Diamond, and D. H. Fremont.** 2005. Structural basis of West Nile virus neutralization by a therapeutic antibody. Nature **437:**764-9.

70. **Oliphant, T., M. Engle, G. E. Nybakken, C. Doane, S. Johnson, L. Huang, S. Gorlatov, E. Mehlhop, A. Marri, K. M. Chung, G. D. Ebel, L. D. Kramer, D. H. Fremont, and M. S. Diamond.** 2005. Development of a humanized monoclonal antibody with therapeutic potential against West Nile virus. Nat Med **11:**522-30.

71. **Oliphant, T., G. E. Nybakken, S. K. Austin, Q. Xu, J. Bramson, M. Loeb, M. Throsby, D. H. Fremont, T. C. Pierson, and M. S. Diamond.** 2007. Induction of epitope-specific neutralizing antibodies against West Nile virus. J Virol **81:**11828-39.

72. **Oliphant, T., G. E. Nybakken, M. Engle, Q. Xu, C. A. Nelson, S. Sukupolvi-Petty, A. Marri, B. E. Lachmi, U. Olshevsky, D. H. Fremont, T. C. Pierson, and M. S. Diamond.** 2006. Antibody recognition and neutralization determinants on domains I and II of West Nile Virus envelope protein. J Virol **80:**12149-59.

73. **Papa, A., K. Danis, A. Baka, A. Bakas, G. Dougas, T. Lytras, G. Theocharopoulos, D. Chrysagis, E. Vassiliadou, F. Kamaria, A. Liona, K. Mellou, G. Saroglou, and T. Panagiotopoulos.** Ongoing outbreak of West Nile virus infections in humans in Greece, July - August 2010. Euro Surveill **15**.

74. **Peeters, C. C., A. M. Tenbergen-Meekes, J. T. Poolman, M. Beurret, B. J. Zegers, and G. T. Rijkers.** 1991. Effect of carrier priming on immunogenicity of saccharide-protein conjugate vaccines. Infect Immun **59:**3504-10.

75. **Pierson, T. C., and M. S. Diamond.** 2008. Molecular mechanisms of antibody-mediated neutralisation of flavivirus infection. Expert Rev Mol Med **10:**e12.

76. **Pierson, T. C., Q. Xu, S. Nelson, T. Oliphant, G. E. Nybakken, D. H. Fremont, and M. S. Diamond.** 2007. The stoichiometry of antibody-mediated neutralization and enhancement of West Nile virus infection. Cell Host Microbe **1:**135-45.

77. **Plotkin, S. A., W. A. Orenstein, and P. A. Offit.** 2008. Vaccines 5th edition. (Saunders).

78. **Pugachev, K. V., F. Guirakhoo, and T. P. Monath.** 2005. New developments in flavivirus vaccines with special attention to yellow fever. Curr Opin Infect Dis **18:**387-94.

79. **Rey, F. A., F. X. Heinz, C. Mandl, C. Kunz, and S. C. Harrison.** 1995. The envelope glycoprotein from tick-borne encephalitis virus at 2 A resolution. Nature **375:**291-8.

80. **Robert Putnak, J., B. A. Coller, G. Voss, D. W. Vaughn, D. Clements, I. Peters, G. Bignami, H. S. Houng, R. C. Chen, D. A. Barvir, J. Seriwatana, S. Cayphas, N. Garcon, D. Gheysen, N. Kanesa-Thasan, M. McDonell, T. Humphreys, K. H. Eckels, J. P. Prieels, and B. L. Innis.** 2005. An evaluation of dengue type-2 inactivated, recombinant subunit, and live-attenuated vaccine candidates in the rhesus macaque model. Vaccine **23:**4442-52.

81. **Rodenhuis-Zybert, I. A., H. M. van der Schaar, J. M. da Silva Voorham, H. van der Ende-Metselaar, H. Y. Lei, J. Wilschut, and J. M. Smit.** Immature dengue virus: a veiled pathogen? PLoS Pathog **6:**e1000718.

82. **Roehrig, J. T.** 2003. Antigenic structure of flavivirus proteins. Adv Virus Res **59:**141-75.

83. **Roehrig, J. T., R. A. Bolin, and R. G. Kelly.** 1998. Monoclonal antibody mapping of the envelope glycoprotein of the dengue 2 virus, Jamaica. Virology **246:**317-28.

84. **Roehrig, J. T., K. E. Volpe, J. Squires, A. R. Hunt, B. S. Davis, and G. J. Chang.** 2004. Contribution of disulfide bridging to epitope expression of the dengue type 2 virus envelope glycoprotein. J Virol **78:**2648-52.

85. **Samuel, M. A., H. Wang, V. Siddharthan, J. D. Morrey, and M. S. Diamond.** 2007. Axonal transport mediates West Nile virus entry into the central nervous system and induces acute flaccid paralysis. Proc Natl Acad Sci U S A **104:**17140-5.

86. **Sanchez, M. D., T. C. Pierson, M. M. Degrace, L. M. Mattei, S. L. Hanna, F. Del Piero, and R. W. Doms.** 2007. The neutralizing antibody response against West Nile virus in naturally infected horses. Virology **359:**336-48.

87. **Sanchez, M. D., T. C. Pierson, D. McAllister, S. L. Hanna, B. A. Puffer, L. E. Valentine, M. M. Murtadha, J. A. Hoxie, and R. W. Doms.** 2005. Characterization of neutralizing antibodies to West Nile virus. Virology **336:**70-82.

88. **Simmons, M., G. S. Murphy, T. Kochel, K. Raviprakash, and C. G. Hayes.** 2001. Characterization of antibody responses to combinations of a dengue-2 DNA and dengue-2 recombinant subunit vaccine. Am J Trop Med Hyg **65:**420-6.

89. **Simmons, M., W. M. Nelson, S. J. Wu, and C. G. Hayes.** 1998. Evaluation of the protective efficacy of a recombinant dengue envelope B domain fusion protein against dengue 2 virus infection in mice. Am J Trop Med Hyg **58:**655-62.

90. **Simmons, M., K. R. Porter, C. G. Hayes, D. W. Vaughn, and R. Putnak.** 2006. Characterization of antibody responses to combinations of a dengue virus type 2 DNA vaccine and two dengue virus type 2 protein vaccines in rhesus macaques. J Virol **80:**9577-85.

91. **Smithburn KC, H. T., Burke AW, Paul JH.** 1940. A neurotropic virus isolated from the blood of a native of Ugande. Am J Trop Med Hyg **20:**471-492.

92. **Spohn, G., G. T. Jennings, B. E. Martina, I. Keller, M. Beck, P. Pumpens, A. D. Osterhaus, and M. F. Bachmann.** 2010. A VLP-based vaccine targeting domain III of the West Nile virus E protein protects from lethal infection in mice. Virol J **7:**146.

93. **Stiasny, K., and F. X. Heinz.** 2006. Flavivirus membrane fusion. J Gen Virol **87:**2755-66.

94. **Stiasny, K., S. Kiermayr, H. Holzmann, and F. X. Heinz.** 2006. Cryptic properties of a cluster of dominant flavivirus cross-reactive antigenic sites. J Virol **80:**9557-68.

95. **Sukupolvi-Petty, S., S. K. Austin, M. Engle, J. D. Brien, K. A. Dowd, K. L. Williams, S. Johnson, R. Rico-Hesse, E. Harris, T. C. Pierson, D. H. Fremont, and M. S. Diamond.** Structure and function analysis of therapeutic monoclonal antibodies against dengue virus type 2. J Virol **84:**9227-39.

96. **Sukupolvi-Petty, S., S. K. Austin, W. E. Purtha, T. Oliphant, G. E. Nybakken, J. J. Schlesinger, J. T. Roehrig, G. D. Gromowski, A. D. Barrett, D. H. Fremont, and M. S. Diamond.** 2007. Type- and subcomplex-specific neutralizing antibodies against domain III of dengue virus type 2 envelope protein recognize adjacent epitopes. J Virol **81:**12816-26.

97. **Thompson, B. S., B. Moesker, J. M. Smit, J. Wilschut, M. S. Diamond, and D. H. Fremont.** 2009. A therapeutic antibody against west nile virus

neutralizes infection by blocking fusion within endosomes. PLoS Pathog **5:**e1000453.

98. **Throsby, M., C. Geuijen, J. Goudsmit, A. Q. Bakker, J. Korimbocus, R. A. Kramer, M. Clijsters-van der Horst, M. de Jong, M. Jongeneelen, S. Thijsse, R. Smit, T. J. Visser, N. Bijl, W. E. Marissen, M. Loeb, D. J. Kelvin, W. Preiser, J. ter Meulen, and J. de Kruif.** 2006. Isolation and characterization of human monoclonal antibodies from individuals infected with West Nile Virus. J Virol **80:**6982-92.

99. **Valdes, I., L. Hermida, L. Gil, L. Lazo, J. Castro, J. Martin, L. Bernardo, C. Lopez, O. Niebla, T. Menendez, Y. Romero, J. Sanchez, M. G. Guzman, and G. Guillen.** 2010. Heterologous prime-boost strategy in non-human primates combining the infective dengue virus and a recombinant protein in a formulation suitable for human use. Int J Infect Dis **14:**e377-83.

100. **Valdes, I., L. Hermida, A. Zulueta, J. Martin, R. Silva, M. Alvarez, M. G. Guzman, and G. Guillen.** 2007. Expression in Pichia pastoris and immunological evaluation of a truncated Dengue envelope protein. Mol Biotechnol **35:**23-30.

101. **Vogt, M. R., B. Moesker, J. Goudsmit, M. Jongeneelen, S. K. Austin, T. Oliphant, S. Nelson, T. C. Pierson, J. Wilschut, M. Throsby, and M. S. Diamond.** 2009. Human monoclonal antibodies against West Nile virus induced by natural infection neutralize at a postattachment step. J Virol **83:**6494-507.

102. **Volk, D. E., Y. C. Lee, X. Li, V. Thiviyanathan, G. D. Gromowski, L. Li, A. R. Lamb, D. W. Beasley, A. D. Barrett, and D. G. Gorenstein.** 2007. Solution structure of the envelope protein domain III of dengue-4 virus. Virology **364:**147-54.

103. **Volk, D. E., F. J. May, S. H. Gandham, A. Anderson, J. J. Von Lindern, D. W. Beasley, A. D. Barrett, and D. G. Gorenstein.** 2009. Structure of yellow fever virus envelope protein domain III. Virology **394:**12-8.

104. **Wahala, W. M., A. A. Kraus, L. B. Haymore, M. A. Accavitti-Loper, and A. M. de Silva.** 2009. Dengue virus neutralization by human immune sera: role of envelope protein domain III-reactive antibody. Virology **392:**103-13.

105. **Watts, D. M., R. B. Tesh, M. Siirin, A. T. Rosa, P. C. Newman, D. E. Clements, S. Ogata, B. A. Coller, C. Weeks-Levy, and M. M. Lieberman.**

2007. Efficacy and durability of a recombinant subunit West Nile vaccine candidate in protecting hamsters from West Nile encephalitis. Vaccine **25:**2913-8.

106. **Wong, S. J., R. H. Boyle, V. L. Demarest, A. N. Woodmansee, L. D. Kramer, H. Li, M. Drebot, R. A. Koski, E. Fikrig, D. A. Martin, and P. Y. Shi.** 2003. Immunoassay targeting nonstructural protein 5 to differentiate West Nile virus infection from dengue and St. Louis encephalitis virus infections and from flavivirus vaccination. J Clin Microbiol **41:**4217-23.

107. **Wu, K. P., C. W. Wu, Y. P. Tsao, T. W. Kuo, Y. C. Lou, C. W. Lin, S. C. Wu, and J. W. Cheng.** 2003. Structural basis of a flavivirus recognized by its neutralizing antibody: solution structure of the domain III of the Japanese encephalitis virus envelope protein. J Biol Chem **278:**46007-13.

108. **Wu, S. C., C. H. Yu, C. W. Lin, and I. M. Chu.** 2003. The domain III fragment of Japanese encephalitis virus envelope protein: mouse immunogenicity and liposome adjuvanticity. Vaccine **21:**2516-22.

109. **Yu, I. M., H. A. Holdaway, P. R. Chipman, R. J. Kuhn, M. G. Rossmann, and J. Chen.** 2009. Association of the pr peptides with dengue virus at acidic pH blocks membrane fusion. J Virol **83:**12101-7.

110. **Zhang, W., P. R. Chipman, J. Corver, P. R. Johnson, Y. Zhang, S. Mukhopadhyay, T. S. Baker, J. H. Strauss, M. G. Rossmann, and R. J. Kuhn.** 2003. Visualization of membrane protein domains by cryo-electron microscopy of dengue virus. Nat Struct Biol **10:**907-12.

6. Outlook

The key finding of this thesis was that not only the native conformation but also the quaternary organisation of the immunogen was a decisive factor for the induction of a potent virus neutralizing antibody response. Our findings demonstrate that, due to the tight packing of E in the virion, the inactivated virus appears to present primarily those antigenic surfaces (on DIII) that are a target of potent neutralizing antibodies. In contrast, isolated recombinant antigens (DIII) display antigenic determinants which are not exposed on the surface of the viral particle and thus induce antibodies which contribute less to neutralization. However, despite its low potency as a priming antigen, the isolated DIII was able to strongly booster the most potent neutralizing antibodies induced by the inactivated virion.

It remains a key question whether the findings obtained in the mouse system can be generalized to other species, especially humans. In previous studies, a significant degree of heterogeneity was observed with respect to fine-specificities of antibody responses in mouse, horse and human post-infection sera. Consistent with our work, antibodies specific for DIII made up a significant proportion of the IgG response in mice, but only a small proportion in humans and horses. In contrast, FP loop-specific antibodies were dominant in humans but represented only a minor fraction in mice, again consistent with our results. Furthermore, it was shown for both, mice and humans that the antibody responses can be subject to strong individual variations. Therefore, it will be important to conduct more detailed studies on fine-specificity, immunodominance and functional activity, especially in human post-infection/post vaccination sera.

Besides native conformation and quaternary organization of the immunogen, the specificity of antibody responses could also be influenced by other factors. For instance, it is not clear whether the genetic background of the host can influence fine-specificity, immunodominance and individual variation of antibody specificity.

Studies adressing these questions can increase our knowledge of factors that control or influence determinants of immunogenicity, fine-specificity and immunodominance of antibody responses. A better understanding of these factors can improve strategies for the rational design of vaccines with higher immunogenicity and protective potency.

7. Curriculum vitae

Personal Information

Date of birth: 1979-07-02
Place of birth: St.Pölten, Austria
Nationality: Austrian

Education

1985-1993	Elementary school in St.Pölten
1993-1997	High school (Main focus on sciences and mathematics) in St.Pölten
1998-2004	Study: Microbiology at the University of Vienna
2003-2004	Diploma thesis performed at the University of Vienna, Institute of Medical Biochemistry; Title: “Consequences of CSF-1 inhibition”.
2005-2010	PhD thesis performed at the Medical University of Vienna, Department of Virology under the supervision of Prof. Franz Xaver Heinz. Title: Fine-specificity and functional quality of the antibody response in mice to whole inactivated West Nile virus and recombinant subunit antigens

Additional experience

Aug-Sep 2002	Internship at “Ökolab Gesellschaft für Umweltanalytik, GmbH” Detection of genetically modified organisms in food products.
March 2007	GV-SOLAS category B certified course at the University of Veterinary Medicine, Vienna: Performing animal experiments (mice, rats, and rabbits).