Development of a humanized monoclonal antibody with therapeutic potential against West Nile virus

Theodore Oliphant[1], Michael Engle[2], Grant E Nybakken[3], Chris Doane[2], Syd Johnson[4], Ling Huang[4], Sergey Gorlatov[4], Erin Mehlhop[3], Anantha Marri[2], Kyung Min Chung[2], Gregory D Ebel[5], Laura D Kramer[5], Daved H Fremont[3] & Michael S Diamond[1,2,3]

Neutralization of West Nile virus (WNV) *in vivo* correlates with the development of an antibody response against the viral envelope (E) protein. Using random mutagenesis and yeast surface display, we defined individual contact residues of 14 newly generated monoclonal antibodies against domain III of the WNV E protein. Monoclonal antibodies that strongly neutralized WNV localized to a surface patch on the lateral face of domain III. Convalescent antibodies from individuals who had recovered from WNV infection also detected this epitope. One monoclonal antibody, E16, neutralized 10 different strains *in vitro*, and showed therapeutic efficacy in mice, even when administered as a single dose 5 d after infection. A humanized version of E16 was generated that retained antigen specificity, avidity and neutralizing activity. In postexposure therapeutic trials in mice, a single dose of humanized E16 protected mice against WNV-induced mortality, and may therefore be a viable treatment option against WNV infection in humans.

WNV is a single-stranded, positive-polarity RNA flavivirus that is related to dengue fever, yellow fever, St. Louis, tick-borne and Japanese encephalitis viruses. Humans infected with WNV develop a febrile illness that can progress to meningitis or encephalitis, and the elderly and immunocompromised are at greatest risk for severe disease[1]. At present, treatment is supportive and no vaccine exists for human use.

The innate and adaptive immune responses prevent WNV dissemination to and within the central nervous system (CNS)[2,3]. Recently, two groups showed therapeutic efficacy of immune human γ-globulin in mice infected with WNV[4,5]. Even after virus had spread to the CNS, passive administration of immune heterologous γ-globulin improved survival[5]. In theory, a potently neutralizing monoclonal antibody could have the same or better benefit with a lower dose and improved safety profile.

Most neutralizing antibodies against flaviviruses recognize the envelope (E) protein. In general, virus-specific rather than cross-reactive antibodies have the strongest neutralizing activity *in vitro* and greatest protection *in vivo*[6]. Crystallographic analysis of the soluble ectodomain of flavivirus E proteins has shown three domains[7,8]. Domain I is an eight-stranded β-barrel[7–9] that participates in the conformational changes associated with the acidification in the endosome[10]. Domain II contains 12 β-strands and has roles in dimerization, trimerization and fusion[7,8,10]. Domain III (DIII) adopts an immunoglobulin-like fold, and contains surfaced-exposed loops in the mature virion[11,12], which putatively have a role in receptor attachment[6,8,13,14]. Based on the sequencing of *in vitro*

neutralization escape variants, many neutralizing antibodies against flaviviruses localize to DIII[15–22].

Here, we define further the molecular basis of antibody-mediated neutralization of WNV using a large panel of newly generated monoclonal antibodies against WNV E protein. Humanized versions of one of these, E16, retained antigen specificity, avidity and neutralizing activity and protected mice against WNV-induced mortality.

RESULTS

Generation of monoclonal antibodies against WNV E protein

Postexposure treatment with neutralizing polyclonal human γ-globulin partially protects mice against WNV[5]. Although human γ-globulin has potential as an immunotherapy for WNV infection, it has several limitations: (i) it is derived from nonimmune and immune donors and has only a modest specific neutralizing titer[5]; (ii) batch variability may affect the efficacy of specific preparations; and (iii) as a human blood product, it has an inherent risk of transmitting infectious agents. To overcome these limitations, we developed a panel of mouse monoclonal antibodies against WNV and determined the *in vitro* and *in vivo* inhibitory potency as a guide for identifying candidates for humanization.

We fused the first 1,290 nucleotides of WNV E protein upstream of a histidine repeat in a baculovirus shuttle vector. The resultant truncated E protein lacked the 71 C-terminal amino acids that correspond to the transmembrane and cytoplasmic regions. We generated recombinant baculoviruses, infected Hi-5 insect cells and purified soluble

Departments of [1]Molecular Microbiology, [2]Medicine and [3]Pathology & Immunology, Washington University School of Medicine, 660 South Euclid Avenue, Box 8051, St. Louis, Missouri 63110, USA. [4]MacroGenics, 1500 East Guide Drive, Rockville, Maryland 20850, USA. [5]Wadsworth Center, New York State Department of Health, 5668 State Farm Road, Slingerlands, New York 12159, USA. Correspondence should be addressed to M.S.D. (diamond@borcim.wustl.edu).
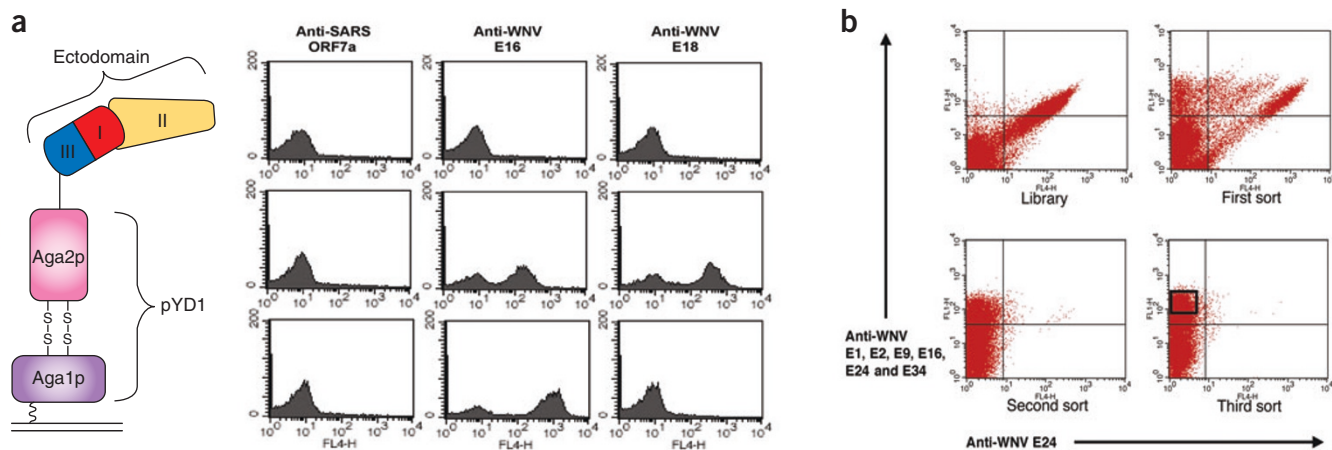
Figure 1 Mapping of monoclonal antibodies to DIII with yeast. (**a**) Surface display of WNV E protein on yeast. A fusion protein is composed of the ectodomain or DIII of WNV E protein and the yeast Aga2 protein, which becomes attached to the Aga1 protein on the yeast cell wall. Yeast were transformed with the vector alone (pYD1; top row), the entire WNV E ectodomain (amino acids 1–415; middle row), or DIII alone (amino acids 296–415; bottom row). 24 h after induction, yeast were stained with the indicated monoclonal antibodies (negative control, anti-SARS ORF7a) and processed by flow cytometry. Data for a representative neutralizing (E16) and non-neutralizing (E18) antibody are shown. (**b**) Flow cytometric enrichment for DIII-expressing yeast variants that lose binding of E24. After each round, an increased percentage of DIII expressing yeast are recognized by the polyclonal WNV E–specific antibody but not by E24. After the final round, DIII-expressing variants (boxed region) were harvested.

E protein by nickel-affinity chromatography (data not shown). After immunization and screening 2,000 hybridomas, we isolated 46 new monoclonal antibodies that recognized WNV E protein (**Supplementary Table 1** online).

Neutralizing activity *in vitro*

We evaluated the antibodies for their ability to block WNV infection in BHK21 cells using a standard plaque-reduction assay[23]. Twelve had strong neutralizing activity that greatly exceeded the potency of immune human γ-globulin, with 50% plaque reduction neutralization titers ($PRNT_{50}$) below 2 µg, whereas immune human γ-globulin had a $PRNT_{50}$ value of 500 µg[5]. The inhibitory activity of two neutralizing antibodies, E16 and E24, was reproduced in J774.2 mouse macrophages and SW13 human adrenal carcinoma cells

(**Supplementary Fig. 1** online) and thus was not specific to fibroblasts.

One of the potent neutralizing monoclonal antibodies, E16, inhibited infection of genetically diverse WNV lineage I strains that were isolated from mosquitoes, birds and horses in New York. E16 neutralized all WNV strains with $PRNT_{50}$ values of 4–18 ng and $PRNT_{90}$ values of 53–297 ng (**Supplementary Table 2** online). Notably, Fab fragments of E16 inhibited WNV ($PRNT_{50}$, 23 ng), suggesting that neutralization does not require bivalent E protein binding. E16 potently blocked infection with strain 956, the original lineage II strain isolated in 1937 (ref. 24), yet was virus specific, as it neither recognized nor neutralized other flaviviruses including distantly related dengue and yellow fever viruses (data not shown) and closely related Japanese and St. Louis encephalitis viruses (**Supplementary Table 2** online).
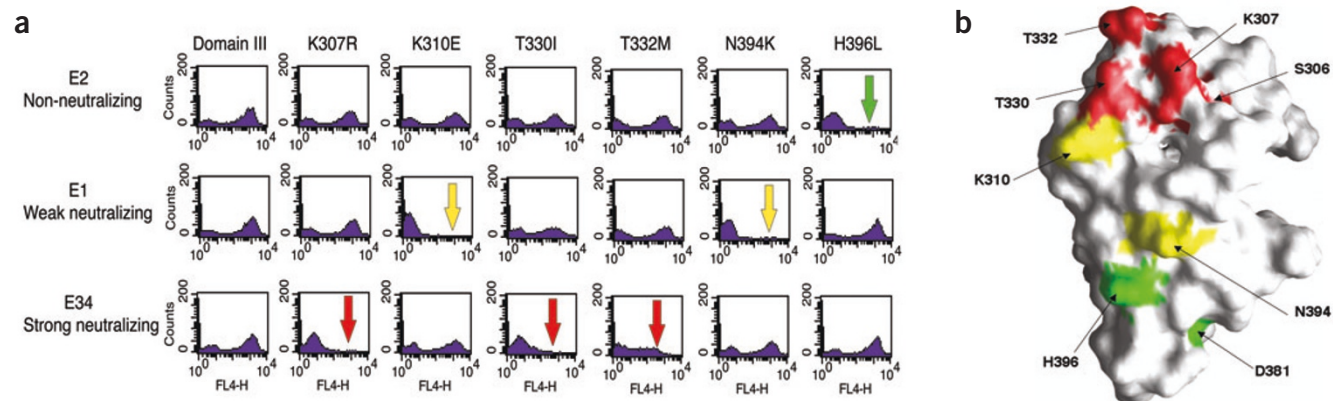

Figure 2 Fine epitope mapping of DIII neutralizing and non-neutralizing monoclonal antibodies. (**a**) Flow cytometry profiles for immunoreactivity by non-neutralizing (E2), weakly neutralizing (E1) and strongly neutralizing (E34) monoclonal antibodies with yeast expressing wild-type and variant DIII. The red, yellow and green arrows point to mutations that abolish yeast surface binding of individual monoclonal antibodies and correspond to distinct regions of DIII (see **b**). (**b**) Mapping of neutralizing and non-neutralizing monoclonal antibodies on the surface of WNV DIII. A molecular surface representation is depicted based on the nuclear magnetic resonance structure of WNV DIII[16]. The indicated amino acid residues associated with binding of neutralizing, weakly neutralizing and non-neutralizing monoclonal antibodies are shown in red, yellow and green, respectively.

ARTICLES

Table 1 Binding of monoclonal antibodies to domain III variants on the surface of yeast

	Wild-type	S306L	K307R	K307N	K307E	K310E	P315R	T330I	T332M	T332V	T332A	N394I	N394K	D381G	H396Y	H396L
Non-neutralizing																
E2	79 (845)	66 (698)	71 (776)	67 (499)	75 (672)	65 (1261)	62 (394)	62 (621)	64 (850)	63 (622)	65 (828)	76 (760)	65 (704)	**18 (<30)**	**12 (<30)**	**9 (<30)**
E9	78 (797)	66 (798)	71 (735)	67 (567)	73 (680)	62 (1334)	60 (288)	63 (495)	63 (843)	63 (612)	65 (716)	77 (784)	64 (666)	74 (174)	71 (161)	71 (139)
E21	78 (880)	66 (770)	71 (899)	68 (680)	75 (754)	66 (1255)	63 (500)	62 (523)	65 (818)	63 (783)	64 (800)	77 (848)	65 (895)	77 (617)	79 (835)	79 (951)
E22	79 (849)	68 (802)	71 (698)	68 (620)	77 (836)	63 (812)	36 (48)	65 (515)	66 (697)	65 (676)	63 (687)	77 (778)	65 (730)	78 (503)	78 (726)	79 (922)
E23	80 (742)	66 (725)	72 (812)	68 (651)	75 (747)	62 (1075)	64 (525)	65 (499)	66 (829)	63 (751)	65 (772)	79 (839)	65 (788)	79 (585)	78 (906)	80 (910)
Weakly neutralizing																
E1	79 (857)	68 (868)	71 (853)	67 (601)	75 (824)	**0 (<20)**	54 (377)	60 (484)	58 (677)	64 (732)	62 (721)	29 (66)	**1 (<20)**	79 (590)	77 (940)	79 (947)
Strongly neutralizing																
E16	78 (656)	34 (63)	50 (82)	36 (59)	**1 (<20)**	62 (757)	59 (394)	**20 (36)**	**12 (119)**	59 (282)	63 (485)	76 (564)	64 (607)	77 (381)	76 (628)	78 (724)
E24	79 (785)	66 (537)	65 (268)	**1 (<20)**	2 (58)	68 (985)	61 (476)	**1 (<20)**	35 (94)	23 (59)	**29 (74)**	76 (829)	65 (635)	77 (447)	78 (848)	79 (939)
E27	79 (749)	**1 (<20)**	50 (129)	**1 (<20)**	**0 (<20)**	60 (760)	63 (355)	65 (459)	62 (436)	55 (190)	60 (331)	78 (743)	65 (746)	79 (511)	77 (722)	78 (671)
E33	79 (754)	66 (637)	37 (191)	**1 (<20)**	**0 (<20)**	64 (718)	58 (376)	**2 (<30)**	54 (174)	60 (321)	**36 (76)**	77 (680)	63 (695)	76 (427)	78 (546)	79 (747)
E34	79 (919)	66 (663)	11 (29)	**0 (<20)**	**1 (<20)**	63 (1113)	64 (624)	**20 (34)**	50 (120)	62 (676)	63 (784)	77 (868)	65 (881)	77 (607)	76 (935)	78 (1035)
E40	79 (622)	43 (116)	13 (32)	11 (33)	**1 (<20)**	64 (733)	66 (401)	43 (79)	56 (180)	62 (398)	61 (366)	77 (545)	64 (623)	76 (402)	76 (663)	79 (582)
E43	79 (648)	24 (52)	**0 (<20)**	**1 (<20)**	**1 (<20)**	61 (691)	62 (439)	**16 (35)**	32 (73)	63 (400)	60 (339)	77 (552)	64 (596)	76 (407)	76 (516)	79 (768)
E47	79 (684)	62 (279)	**0 (<20)**	**1 (<20)**	**1 (<20)**	61 (674)	66 (413)	**1 (<20)**	49 (148)	64 (502)	64 (625)	77 (760)	64 (716)	77 (446)	77 (387)	79 (730)
E49	76 (409)	**18 (52)**	**0 (<20)**	**0 (<20)**	**0 (<20)**	49 (116)	53 (93)	**2 (<20)**	**4 (38)**	64 (556)	65 (687)	74 (373)	58 (265)	**64 (113)**	75 (323)	78 (487)
E58	79 (742)	64 (323)	**2 (<20)**	5 (25)	3 (37)	64 (639)	64 (403)	**7 (65)**	**1 (<20)**	**4 (<20)**	**1 (<20)**	77 (746)	63 (728)	76 (425)	77 (671)	79 (727)

Individual WNV-specific monoclonal antibodies (25 µg/ml) were mixed with yeast that displayed wild-type or mutant DIII on their surface. After washing, and incubation with an Alexa-647 goat-anti mouse IgG secondary antibody, yeast were analyzed for antibody binding by flow cytometry. The two values represent the percentage of yeast that were positive for DIII expression with a given monoclonal antibody, and in parentheses, the mean linear fluorescence intensity of the positive cells. Yeast were analyzed at 24 h after induction with galactose, which gives a baseline surface expression of DIII of 60–80% positive cells (**Fig. 1a**). Bold values indicate an almost complete (>80%) loss of binding, whereas underlined values show a marked (50–79%) reduction in either the percentage or the mean fluorescence intensity of the positive cells. Results are representative of at least two independent experiments for each antibody and DIII variant.

Mapping of neutralizing monoclonal antibodies to DIII

To map our strongly neutralizing monoclonal antibodies, we developed a strategy using yeast surface display[25]. The ectodomain (amino acids 1–415) or DIII (amino acids 296–415) of WNV E protein were expressed as fusion proteins on the yeast cell surface (**Fig. 1a**). Monoclonal antibodies that recognize DIII alone are considered DIII specific. Monoclonal antibodies that recognized the E ectodomain but not DIII alone may contact residues that map to domain I or II or both, although cooperative contacts with DIII cannot be ruled out. Most of our 46 monoclonal antibodies recognized yeast that displayed the entire ectodomain of E (**Fig. 1a** and **Supplementary Table 1** online). Sixteen antibodies recognized yeast that displayed DIII alone, and 10 of 12 strongly neutralizing antibodies localized to DIII. Only two neutralizing antibodies (E53 and E60) recognized the E ectodomain but not DIII alone.

Mapping of contact residues of DIII monoclonal antibodies

We used error-prone PCR mutagenesis of DIII of WNV E protein and yeast surface expression to map antibody contact residues in a high-throughput manner. We performed individual screens to identify DIII mutants that lost binding selectively to strongly neutralizing (E16, E24 and E34), weakly neutralizing (E1), and non-neutralizing (E2 and E22) monoclonal antibodies. To eliminate mutants that abolished surface expression of DIII, yeast were stained sequentially with an Alexa Fluor 647–conjugated individual monoclonal antibodies and an Alexa Fluor 488–conjugated oligoclonal antibody derived from a pool of individual monoclonal antibodies. After cell sorting, we identified yeast that selectively lost expression of an individual monoclonal antibody epitope but retained surface expression of DIII (**Fig. 1b**). Multiple independent

yeast that lost binding of individual monoclonal antibodies were subjected to plasmid recovery and sequencing.

Monoclonal antibodies that localized to DIII and strongly neutralized WNV had reduced binding when residues S306, K307, T330 or T332 were altered (**Fig. 2a** and **Table 1**). These are located on adjacent loops and form a contiguous patch[16] on the solvent-exposed surface at the lateral tip of the DIII (**Fig. 2b**). Only two other mutations caused considerable loss of binding of any of the ten neutralizing monoclonal antibodies tested: K310E or P315R reduced binding only of E49. No two neutralizing monoclonal antibodies had identical loss-of-binding patterns. For example, S306L reduced binding of E16, E27, E40, E43 and E49 but not E24, E33, E34, E47 and E58. K307R abolished binding of E34, E40, E43, E47, E49 and E58 but affected E16, E24, E27 and E33 less strongly. In contrast, K307E decreased binding of all neutralizing monoclonal antibodies yet did not affect non-neutralizing or poorly neutralizing monoclonal antibodies. Changes in residues T330 and T332 also abolished binding of neutralizing but not non-neutralizing monoclonal antibodies. T330I or T332M strongly reduced binding of all neutralizing monoclonal antibodies with the exception of E27, and T332V or T332A weakened binding of only E24, E27, E33 and E58.

Six monoclonal antibodies that recognized DIII were either poorly neutralizing or non-neutralizing, and none engaged the dominant neutralizing epitope defined by S306, K307, T330 or T332. E2 and E9 were abolished or reduced by mutation of D381 and H396, and binding of E22 was weakened by a change in P315. D381 and H396 are proximal to one another but physically distinct from the four residues that affect binding of neutralizing monoclonal antibodies (**Fig. 2b**). None of the mutations identified by loss-of-binding sorts for E2 or E22 had any effect on two other non-neutralizing monoclonal

antibodies, E21 and E23. E1, a monoclonal antibody with weak neutralizing activity, mapped to a site between the non-neutralizing and neutralizing monoclonal antibodies, as mutation of K310 and N394 strongly inhibited binding.

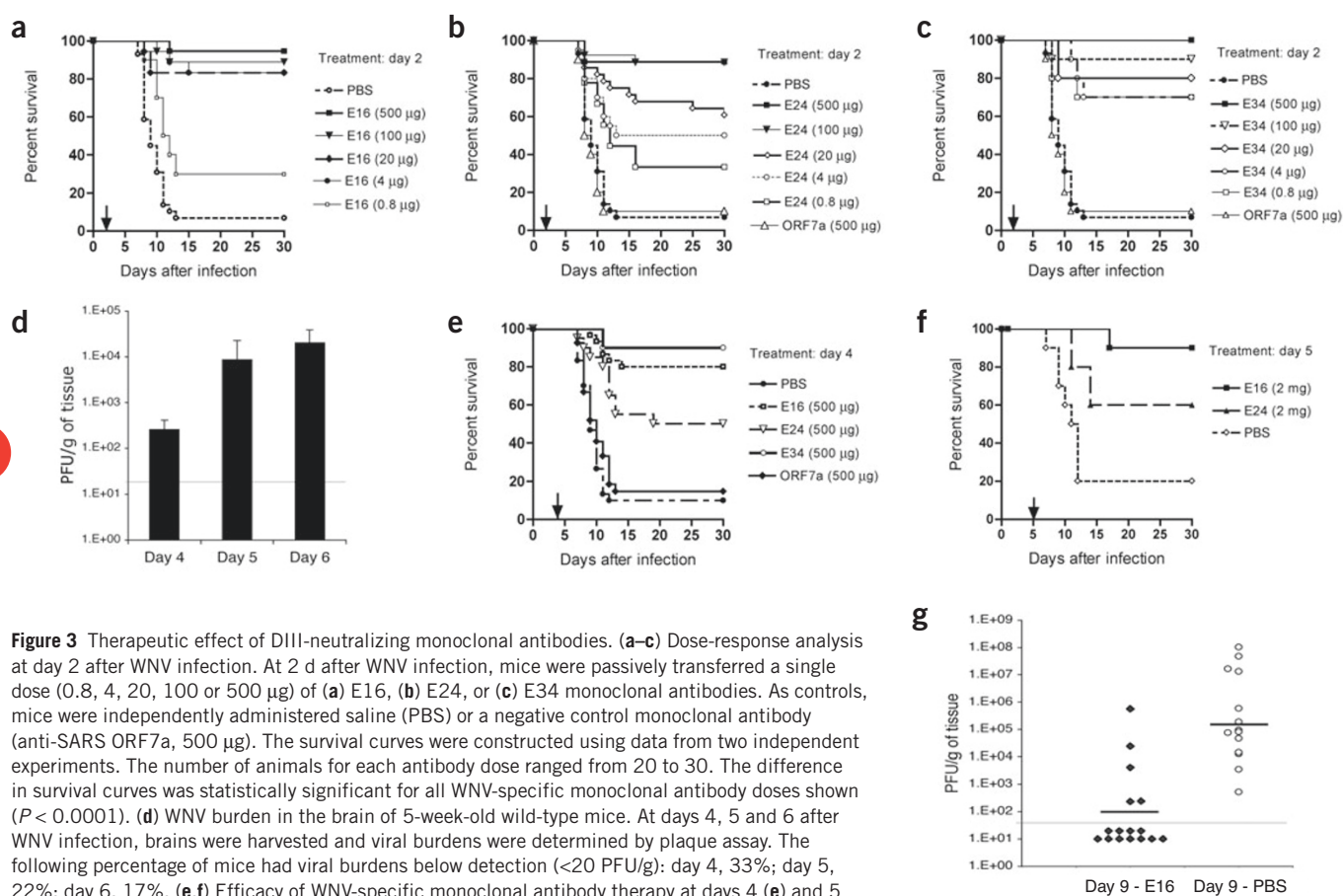### Patient antibodies recognize the dominant neutralizing epitope

To determine whether human antibodies specific for WNV recognize the neutralizing epitope on DIII during infection, plasma was obtained from WNV-positive individuals. Samples from convalescent individuals were negative for WNV RNA but positive for neutralizing WNV-specific antibodies. The individuals reported mild systemic illness, though none progressed to severe disease. To determine whether these samples contained antibodies that localized to the neutralizing epitope on DIII, we tested whether E16 Fab or IgG could compete binding to recombinant, wild-type and mutant N394K and K307N forms of DIII (**Supplementary Fig. 2** online); N394K retains wild-type binding to E16, whereas K307N has markedly reduced binding. E16 equivalently inhibited binding of patient WNV-specific antibodies to wild-type (Fab, 35% ± 8; IgG, 40% ± 12) or N394K DIII (Fab, 32% ± 9; IgG, 34% ± 11), whereas E53 IgG, a WNV-specific monoclonal antibody that recognizes an epitope outside DIII, did not compete binding to wild-type (1% ± 4) or N394K

(−2% ± 5) DIII. As expected, E16, which only weakly recognizes K307N, poorly competed (Fab 6% ± 3; IgG 9% ± 4) binding to K307N DIII. These data suggest that humans, who clear WNV infection, develop antibodies that recognize an epitope in close proximity to that defined by E16.

Therapeutic studies in mice

To evaluate the correlation between neutralization, epitope localization and *in vivo* protection, we assessed the therapeutic activity of different neutralizing monoclonal antibodies in an established mouse model[5]. Studies were performed with 5-week-old wild-type C57BL/6 mice, which have a ~10% survival rate[5]. Mice were inoculated subcutaneously with 10^2 plaque-forming units (PFU) of WNV and administered a single dose of monoclonal antibody at day 2 after infection. Notably, 500 μg of the non-neutralizing monoclonal antibody E2 provided no protection (data not shown). In contrast, 100 μg of any of three different neutralizing monoclonal antibodies that map to K307 (E16, E24 or E34) protected greater than 90% of mice from lethal infection (**Fig. 3a–c**). Even a single 4 μg treatment of E16 or E34 on day 2 after infection prevented mortality.

Given that humans can present with WNV infection of the CNS, we evaluated the therapeutic efficacy of monoclonal antibodies at later

Figure 3 Therapeutic effect of DIII-neutralizing monoclonal antibodies. (**a–c**) Dose-response analysis at day 2 after WNV infection. At 2 d after WNV infection, mice were passively transferred a single dose (0.8, 4, 20, 100 or 500 μg) of (**a**) E16, (**b**) E24, or (**c**) E34 monoclonal antibodies. As controls, mice were independently administered saline (PBS) or a negative control monoclonal antibody (anti-SARS ORF7a, 500 μg). The survival curves were constructed using data from two independent experiments. The number of animals for each antibody dose ranged from 20 to 30. The difference in survival curves was statistically significant for all WNV-specific monoclonal antibody doses shown (*P* < 0.0001). (**d**) WNV burden in the brain of 5-week-old wild-type mice. At days 4, 5 and 6 after WNV infection, brains were harvested and viral burdens were determined by plaque assay. The following percentage of mice had viral burdens below detection (<20 PFU/g): day 4, 33%; day 5, 22%; day 6, 17%. (**e,f**) Efficacy of WNV-specific monoclonal antibody therapy at days 4 (**e**) and 5 (**f**) after infection. A single dose (0.5 mg at day 4 or 2 mg at day 5) of monoclonal antibody (E16, E24, E34 or anti-SARS ORF7a) was administered either 4 or 5 d after WNV infection. Data reflect approximately 20 mice per condition. The difference in survival curves was statistically significant for all WNV-specific monoclonal antibody doses shown at day 4 (*P* < 0.0001) and day 5 (E16, *P* = 0.0009; E24, *P* = 0.027). (**g**) Effect of E16 therapy on viral burden. Mice were treated with 2 mg of E16 or PBS on day 5 after WNV infection. On day 9, brains were recovered, homogenized and subjected to plaque assay. For a subset (6) that received PBS treatment, brains were harvested at days 7 and 8 from moribund mice. The data is expressed as PFU/g. Of 16 mice treated with E16 68% (11) had undetectable viral loads in the brain at day 9 whereas all mice (14 of 14) treated with PBS had detectable virus at the time of harvest. The dotted line represents the limit of sensitivity of the assay and the dark bars represent the mean of the log values. The differences were statistically different (*P* < 0.0001).

time points. At days 4, 5 and 6 after WNV infection, we detected virus in the brains of 67%, 78% and 83% of mice, respectively (**Fig. 3d**). A single 500 μg dose of E16 or E34 at day 4 resulted in an 80–90% survival rate (**Fig. 3e**). A single 2 mg dose of E16 at day 5 resulted in 90% survival (**Fig. 3f**) and complete clearance of WNV from the brain in 68% of mice by day 9 (**Fig. 3g**). Thus, administration of neutralizing monoclonal antibodies to mice with active CNS infection improved survival and induced a virologic cure. As expected, lengthening the interval before treatment was associated with decreased benefit. Administration of E16 at day 6 did not enhance survival (data not shown), although average survival time was increased (9.5 d ± 0.4 to 11.5 d ± 0.4; $P = 0.003$).

Humanization of E16

We considered humanizing E16 or E34 as a possible therapeutic measure. Humanized monoclonal antibodies have substantially longer half-lives in humans than their mouse counterparts[26,27]. Sequencing studies indicated that E16 had greater homology to human framework regions, making it simpler to construct a humanized version of E16.

We amplified the cDNA encoding the heavy (VH) and light (VL) variable domains from the hybridoma cellular RNA by a 5′ rapid amplification of cDNA ends (RACE) procedure. The VH belongs to mouse heavy chain subgroup II (J558 family) and the VL belongs to mouse κ chain subgroup V. The complementarity-determining regions of E16 were grafted onto the human VH1–18 backbone (**Fig. 4a**) and human Vκ-B3 backbone (**Fig. 4b**) to create Hm-E16.1. One (VL-Y49S) and two framework back-mutations (VH-T71A and VL-Y49S) were introduced to create two variants, Hm-E16.1 and Hm-E16.3, respectively. The resulting humanized VH and VL were combined with human γ1 and κ constant regions, fused to an IgG signal sequence and inserted into expression plasmids. To construct the chimerized antibodies, the mouse VH and VL sequences were combined with the human γ1 and κ constant regions.

We expressed humanized (Hm-E16) and chimerized (Ch-E16) E16 in HEK-293 cells, and purified them from supernatants by affinity and size-exclusion chromatography (data not shown). The affinity was analyzed by surface plasmon resonance using purified antibody in the solid phase. Mouse E16 binds DIII with an affinity of 3.4 nM and a half-life of 3.9 min. The affinity of the Ch-E16 and Hm-E16 was similar with K_D ranging from 7.1 to 21 nM (**Fig. 4c**). Hm-E16, Ch-E16, and the parent E16 all had similar PRNT$_{50}$ values (**Fig. 4d**).

We hypothesized that E16 could also control infection in mice through effector functions including antibody-dependent complement fixation and cytotoxicity. To test this, we generated a Ch-E16 N297Q aglycosyl variant that neutralizes WNV (**Fig. 4d**) but does not
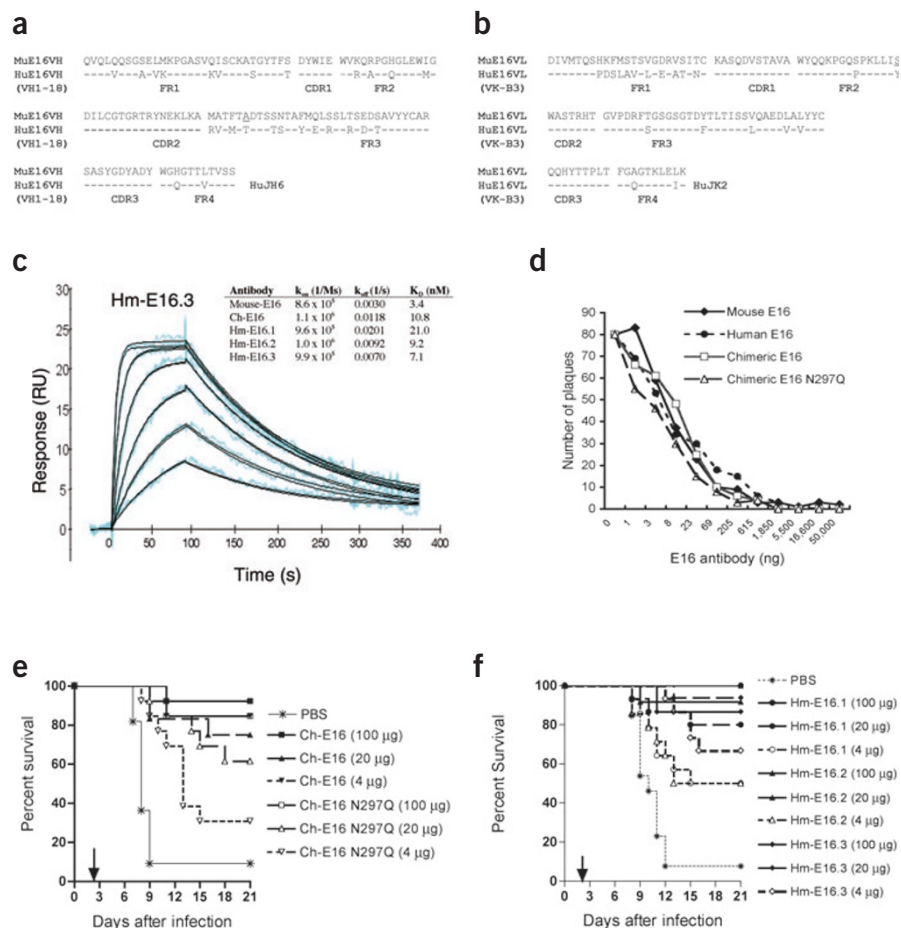
Figure 4 Construction and efficacy of humanized E16. (**a,b**) Sequence and alignment of VH and VL of mouse and humanized E16 (Hm-E16.1). Residues VL-49 and VH-71 were selected for back-mutation in version 16.2 (VL-Y49L) and 16.3 (VL-Y49L, VH-T71A) from the human to the mouse sequence to improve avidity and potency. (**c**) Binding curves and kinetic parameters of DIII binding to chimeric and humanized E16. One representative surface plasmon resonance experiment for Hm-E16.3 is shown. (**d**) Neutralization studies with different E16 antibodies. Neutralizing activity was determined by a PRNT assay. The indicated amount of mouse, humanized, chimerized or aglycosyl chimerized E16 were mixed with WNV before addition to BHK-21 cells. Samples were performed in duplicate and the experiment is one representative of three. (**e**) Therapeutic activity of Ch-E16 and aglycosyl Ch-E16. At 2 d after WNV infection, mice were passively transferred saline or a single intraperitoneal inoculation of increasing doses (4, 20 or 100 μg) of Ch-E16 or aglycosyl Ch-E16 N297Q that differs by a single amino acid. The survival curves were constructed using data from two independent experiments with 13 mice for each arm. At the 4 μg dose, the survival difference between Ch-E16 and aglycosyl Ch-E16 was statistically different ($P = 0.008$). (**f**) Therapeutic activity of Hm-E16 in wild-type mice. At 2 d after WNV infection, mice were passively transferred saline, or a single dose (4, 20 or 100 μg) of three versions of humanized E16 (16.1, 16.2 or 16.3). The survival curves were constructed using data from two or three independent experiments with at least 15 animals per treatment arm. All doses of Hm-E16 provided statistically significant protection compared to treatment with saline alone ($P < 0.007$).

efficiently fix complement or bind Fc γ receptors[28]. Mice were administered Ch-E16 or Ch-E16 N297Q at day 2 after WNV infection. Although high doses of Ch-E16 and Ch-E16 N297Q provided virtually complete protection, lower doses of the aglycosyl variant afforded less protection (**Fig. 4e**). Administration of 4 μg of Ch-E16 resulted in 84% survival, whereas 4 μg of Ch-E16 N297Q provided only 31% protection.

To test which effector function enhanced the activity of E16, we performed studies with 8-week-old *C1qa*, *C4*, or *Fcgr1* and *Fcgr3*-deficient C57BL/6 mice (**Fig. 5**). These mice all show increased susceptibility to lethal WNV infection compared to wild-type controls. In *C1qa* or
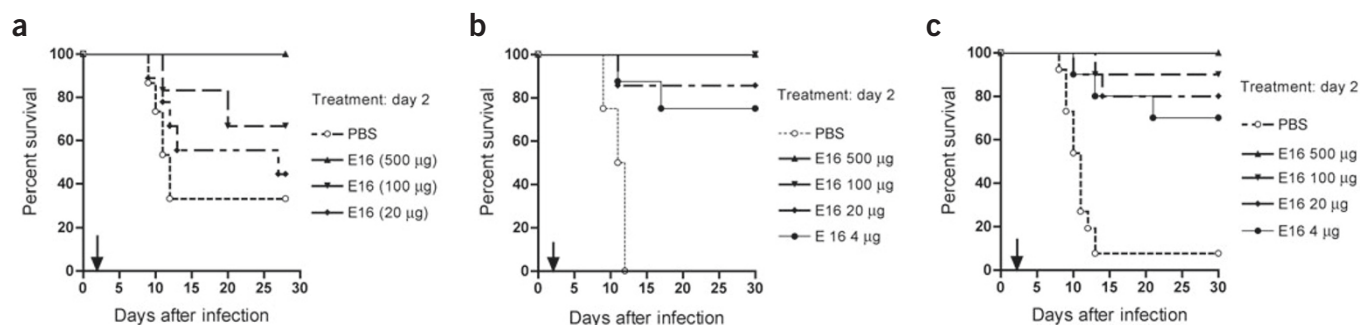
Figure 5 Efficacy of mouse E16 in complement and *Fcgr1*- and *Fcgr3*-deficient mice. Dose-response analysis at day 2 after WNV infection. (**a**) *Fcgr1*- and *Fcgr3*-deficient, (**b**) *C1qa*-deficient, or (**c**) *C4*-deficient 8-week-old C57BL/6J mice were inoculated with 10^2 PFU of WNV. At 2 d after infection, mice were passively transferred a single dose (4, 20, 100 or 500 μg) of mouse E16. As controls, mice were independently administered saline (PBS). The number of animals for each antibody dose ranged from 10 to 15. The difference in survival curves was statistically significant for all WNV-specific monoclonal antibody doses shown in the *C1qa*- and *C4*-deficient mice ($P \leq 0.001$). The difference in survival curves for the *Fcgr1*- and *Fcgr3*-deficient mice was significant for 500 μg ($P = 0.01$), but not for 100 μg ($P = 0.14$) or 20 μg ($P = 0.4$) of mouse E16.

C4-deficient mice, which cannot activate complement by the antibody-dependent classical pathway, E16 had a similar potency compared to wild-type mice. In contrast, in *Fcgr1*- and *Fcgr3*-deficient mice, although high doses afforded complete protection, lower doses resulted in higher mortality rates. A dose of 20 μg at day 2, which strongly protected wild-type, *C1qa* or *C4*-deficient mice, did not improve the survival rate of *Fcgr1*- and *Fcgr3*-deficient mice ($P = 0.4$). Thus, the Fc region enhances the potency of E16 in mice, by virtue of its ability to bind to Fc γ receptors.

To confirm the efficacy of humanized E16, wild-type mice were administered three different versions of purified Hm-E16 at day 2 after infection. Although Hm-E16 variants protected mice against lethal infection (**Fig. 4f**), at a dose of 4 μg, the variant (16.3) that showed the highest affinity for DIII (**Fig. 4c**) was more protective (67% versus 46% survival; mean survival time of 14 ± 1 d versus 11 ± 2 d, $P = 0.04$).

DISCUSSION

We generated a panel of 46 monoclonal antibodies against WNV E protein, and applied a new high-throughput epitope-mapping strategy to identify a dominant epitope that was recognized by the majority of neutralizing monoclonal antibodies in DIII. This epitope was also detected by convalescent antibodies from individuals who had recovered from WNV infection without clinical consequence. Three neutralizing monoclonal antibodies protected against WNV mortality in a postexposure therapy model. One of these, E16, was humanized and confirmed as therapeutically effective in mice.

Previous studies have mapped amino acid contact residues of neutralizing monoclonal antibodies by sequencing *in vitro* neutralization escape variants, through site-specific substitution of specific charged or polar residues, and by performing binding assays with overlapping peptide libraries. We used error-prone PCR mutagenesis and yeast surface expression to identify contact residues in a high-throughput manner. Although this technique has been used previously[29], this is the first high-throughput epitope-mapping application. By having a large panel of DIII monoclonal antibodies and selecting only variants that abolished or markedly reduced binding of a few monoclonal antibodies, we minimized the possibility that mutations altered protein folding. We have recently confirmed the recognition sites on DIII for E16 and the validity of the yeast display strategy by solving the crystal structure of the E16 Fab-DIII complex (G.N., T.O., M.D, & D.F., unpublished data).

Of 12 neutralizing monoclonal antibodies, 10 localized to the distal lateral surface of DIII, results that are consistent with prior studies

that mapped three neutralizing monoclonal antibodies against WNV using *in vitro* escape variants[15,30]. Our weakly neutralizing and non-neutralizing monoclonal antibodies did not recognize this epitope but localized to distinct regions. E16 recognized the dominant epitope and neutralized all strains that were tested. Sequence analysis of 124 WNV strains in public databases showed almost complete (98.4–100%) conservation of the contact residues S306, K307, T330 and T332. Only two clinically attenuated lineage II isolates had mutations at these residues. Because of the structural homology among flaviviruses[7,8,16], the analogous amino acids that map to the distal lateral surface of DIII can be readily identified. Although this region is highly variable among flaviviruses, most mutations that abolish binding of virus-specific neutralizing antibodies map here[15,18,30,31], suggesting the existence of an analogous dominant neutralizing epitope for other flaviviruses. We speculate that successful vaccines against WNV or other flaviviruses should induce potent humoral responses against this neutralizing epitope.

Although passive administration of immune human γ-globulin after WNV infection improved survival in mice[4,5], it may be limited by its low-titer neutralizing activity, variability and risk of transmission of infectious agents. Only two prior studies have shown postexposure therapy of neutralizing monoclonal antibodies with flaviviruses: 6B5A-2 reduced mortality 3–4 d after infection with St. Louis encephalitis virus[6]; and 503 reduced mortality resulting from infection with Japanese encephalitis virus 5 d[32]. Here, we show that three different neutralizing monoclonal antibodies improved survival even when administered 4 and 5 d after WNV infection. Moreover, therapy with E16 at day 5 completely cleared WNV from the brain at day 9 in 68% of mice. Thus, inhibitory WNV monoclonal antibodies improve clinical and virologic outcome even after viral spread through the CNS, results that agree with studies showing that antibody can mediate viral clearance from infected neurons[33,34].

Our experiments are consistent with a model in which the therapeutic efficacy of monoclonal antibodies is determined by properties in addition to neutralization: (i) the monoclonal antibody (E24) with the strongest neutralizing activity *in vitro* did not have the greatest efficacy *in vivo*; (ii) an aglycosyl version of E16 that lacked the ability to fix complement or bind to Fc γ receptors had equivalent neutralizing but reduced therapeutic activity; (iii) E16 was less potent in mice that lacked Fc γ receptors.

E16 was humanized as a possible therapeutic measure for humans. Hm-E16 bound DIII with similar affinity and showed efficacy as postexposure therapy. Moreover, it may be possible to improve Hm-E16 by

introducing mutations that enhance affinity, creating forms of E16 that more readily cross the blood-brain barrier, and combining monoclonal antibodies that neutralize WNV infection through independent mechanisms. Our results are the first successful demonstration of a humanized monoclonal antibody as postexposure therapy against a viral disease, and suggest that antibody-based therapeutics may have more broad utility than previously appreciated, especially in the treatment of CNS infections in which an effective antibody response is important for limiting virus dissemination and injury to neurons.

METHODS

Cells and viruses. We cultured BHK-21, Vero and C6/36 *Aedes albopictus* cells as previously described[35]. J774.2 mouse macrophages and SW13 human adrenal cortex adenocarcinoma cells were cultured in DMEM supplemented with 10% FBS. The majority of experiments were performed with the WNV strain (3000.0259, passage 2) that was isolated from a *Culex restuans* mosquito in New York in 2000. We performed additional neutralization experiments with lineage I strains from New York from the years 2000–2003 (03001956, 32010157, NYC01035, 03002094, 02002640, 02002831, 02003688, 31000352, 00-7365) and a lineage II strain (956) isolated from a human in patient Uganda in 1937 (refs. 24,36). We also performed neutralization experiments with prototype strains of St. Louis (59268 (Parton)) and Japanese encephalitis (Nakayama) viruses[37]. For *in vivo* experiments, viruses were diluted and injected into mice as described[23].

Purified WNV E protein expression. WNV E protein ectodomain was generated using a baculovirus expression system according to previously described methods for related flaviviruses[38]. The last 45 nucleotides of prM (endogenous signal sequence) and the first 1,290 nucleotides of WNV E protein from the New York 1999 strain[39] were fused downstream of the polyhedrin promoter and upstream of a histidine repeat in a baculovirus shuttle vector (pFastBac, Invitrogen) by PCR using a high-fidelity Taq polymerase (Platinum Taq, Invitrogen). Three days after baculovirus infection of Hi-5 insect cells at a multiplicity of infection (MOI) of 1, supernatants were harvested, filtered, buffer-exchanged and purified by nickel-affinity chromatography according to the manufacturer's instructions. The purified WNV E ectodomain lacks the C-terminal 71 amino acids that are associated with the membrane proximal, transmembrane and cytoplasmic domains.

Purified WNV DIII. The construction, expression, purification and refolding of DIII of WNV E protein is described in greater detail elsewhere (G.N., T.O., M.D. & D.F., unpublished data). Briefly, wild-type, N394K and K307N DIII were generated from an infectious cDNA clone of the New York 1999 strain of WNV (gift of R. Kinney, Centers for Disease Control and Prevention, Fort Collins, CO) using PCR and Quik-change mutagenesis (Stratagene). After cloning into a PET21 vector (Novagen) and sequence confirmation of the mutations, we transformed plasmids into BL21 Codon Plus *E. coli* cells (Stratagene). Bacteria were grown in Luria broth, induced with 0.5 mM isopropyl thiogalactoside (IPTG) and pelleted. Subsequently, we lysed bacteria after the addition of lysozyme, sonicated them and recovered DIII as insoluble aggregate from the inclusion bodies. DIII was denatured in the presence of guanidine hydrochloride and β-mercaptoethanol and refolded by slowly diluting out the denaturing reagents in the presence of L-arginine, EDTA, PMSF, reduced glutathione and oxidized glutathione. We separated refolded DIII from aggregates on a Superdex 75 16/60 size-exclusion column (Amersham Bioscience) and concentrated it using a centricon-10 spin column into 20 mM Hepes pH 7.4, 150 mM NaCl and 0.01% NaN3. After refolding, wild-type DIII reacted with all domain III–specific monoclonal antibodies including those that recognized conformationally sensitive epitopes.

Generation and purification of monoclonal antibodies. BALB/c mice were primed and boosted at 3-week intervals with insect cell–generated, purified, recombinant WNV E protein (25 μg) that was complexed with adjuvant (RIBI Immunochemical). Approximately 1 month after the last boost, we harvested serum and tested it for immunoreactivity against solid-phase purified E. Mice with high titers (>1/10,000) were boosted intravenously with purified E protein (5 μg) in PBS. We harvested splenocytes 3 d later and fused them to P3X63Ag8.653 myeloma cells to generate hybridomas according to published procedures[40]. We purified monoclonal antibodies against WNV or other control antigens by stan-

dard protein A or protein G chromatography according to the manufacturer's instructions (Pharmacia).

Mouse experiments. For WNV infection experiments, all wild-type C57BL/6J mice were purchased from a commercial source (Jackson Laboratories). We obtained the congenic *C1qa*-deficient and *C4*-deficient mice from G. Stahl (Beth Israel Deaconess Medical Center, Boston, MA) and M. Carroll (The CBR Institute for Biomedical Research, Harvard Medical School, Boston, MA), respectively. We obtained the congenic *Fcgr1*- and *Fcgr3*-deficient mice commercially (Taconic). We used mice between 5 and 8 weeks of age depending on the particular experiment and inoculated them subcutaneously with WNV by footpad injection after anesthetization with xylazine and ketamine. Mouse experiments were approved and performed according to the guidelines of the Washington University School of Medicine Animal Safety Committee.

For passive-transfer experiments, we administered to mice a single dose of purified monoclonal antibody by intraperitoneal injection at a given time point (day 2, 4 or 5) after infection. To analyze virus production in the brain, infected mice were killed on a given day after inoculation. After cardiac perfusion with PBS, we removed the brains, weighed and homogenized them, and performed plaque assays as previously described[23].

Expression of WNV E protein on yeast. The ectodomain or DIII of WNV E protein was expressed on the surface of yeast using a modification of a previously described protocol for surface expression of T cell receptors[29]. Amino acid residues 1–415 (ectodomain) or 296–415 (DIII) of WNV E protein were amplified with *Bam*HI and *Xho*I sites at their 5′ and 3′ ends, respectively, by PCR from the New York 1999 infectious cDNA clone (R. Kinney, Centers for Disease Control and Prevention, Fort Collins, CO). The resulting products were digested with *Bam*HI and *Xho*I, and cloned as downstream fusions to the yeast Aga2 and Xpress epitope tag genes in the yeast surface display vector pYD1 (Invitrogen). An upstream GAL1 promoter controls fusion protein expression. These constructs were transfected into the *S. cerevesiae* yeast strain EBY100 (refs. 25,41) resulting in yeast that expressed the WNV E ectodomain or DIII. Yeast that only expressed the Xpress epitope tag linked to Aga2 were prepared in parallel by transfecting EBY100 cells with the parent vector pYD1. Individual yeast colonies were grown to log phase overnight in tryptophan-free media containing 2% glucose at 30 °C and harvested in log phase. Fusion protein expression was induced on the yeast surface by growing yeast for an additional 24 h in tryptophan-free media containing 2% galactose at 25 °C. We harvested yeast, washed them with PBS supplemented with 1 mg/ml BSA and immunostained them with 50 μl of monoclonal antibody (25 μg/ml) against the Xpress tag or WNV E protein. After 30 min, we washed yeast three times and stained them with a goat anti-mouse secondary antibody conjugated to Alexa Fluor 647 (Molecular Probes). Subsequently, the yeast cells were analyzed on a Becton Dickinson FACSCaliber flow cytometer.

Library construction and screening. We mutated DIII of the WNV E protein using an error-prone PCR protocol[25] that included Mn2+ and Mg2+ at concentrations of 0.3 and 2.0 mM respectively. Subsequently, the cDNA library was ligated into pYD1 and transformed into XL2-blue ultracompetent cells (Strategene). The colonies were pooled and the plasmid DNA was recovered using the Qiagen HiSpeed Maxi kit.

For each individual antibody, we screened the yeast library of DIII mutants according to the following protocol. To identify yeast that selectively lost binding to a given monoclonal antibody epitope, the library was initially stained with an Alexa Fluor 647–conjugated WNV-specific monoclonal antibody for 30 min at 4 °C. To control for the surface expression of DIII, after washing, yeast were subsequently stained for 30 min at 4 °C with an Alexa Fluor 488–conjugated oligoclonal antibody that was derived from a pool of individual monoclonal antibodies (E1, E2, E9, E16, E24 and E34). After immunostaining, we subjected yeast to flow cytometry and identified the population that was single monoclonal antibody negative but pooled oligoclonal antibody positive. The yeast cells were sorted at an event rate of ~4,000 cells/s and this population (monoclonal antibody–negative and oligoclonal antibody–positive) was enriched after three rounds of sorting. After the final enrichment sort, we plated yeast and selected individual colonies and tested them for binding to individual monoclonal antibodies. For individual clones that had lost only the desired monoclonal antibody epitope, the DIII-pYD1

plasmid was recovered using the Zymoprep Yeast Miniprep kit (Zymo Research). The plasmid was then transformed into DH5α cells, purified using the Qiaprep Spin Miniprep kit (Qiagen) and sequenced.

In some cases, DIII variants with two independent mutations were isolated. To determine which mutation conferred the loss-of-binding phenotype, single independent mutations were engineered by site-directed mutagenesis of DIII-pYD1 using mutant oligonucleotides and the Quik Change II mutagenesis kit (Stratagene). All mutations were confirmed by sequencing.

Quantification and characterization of neutralizing antibodies *in vitro*. We determined the titer of neutralizing antibodies by a standard plaque reduction neutralization titer (PRNT) assay using either BHK21 or SW13 cells[23]. Results were plotted and the titers for 50% ($PRNT_{50}$) and 90% inhibition ($PRNT_{90}$) were calculated. The inhibition assay with J774.2 mouse macrophages was performed as follows: we mixed medium and E16 or E24 (2.5 µg of monoclonal antibody) with 5×10^2 PFU of WNV, incubated the mixture for 1 h at 4 °C, and then added to 5×10^4 J774.2 mouse macrophages in individual wells of a 24-well plate. After 1 h, cells were washed four times with PBS to remove free virus and monoclonal antibody, DMEM with 10% FBS was added, and the cells were incubated for an additional 24 h. We subsequently harvested supernatants for a viral plaque assay on Vero cells.

Competition ELISA with human anti-WNV antibodies. After purification and refolding, wild-type, K307N and N394K DIII were diluted (5 µg/ml) in 0.1 M Na carbonate buffer (pH 9.3) and adsorbed to 96-well plates overnight at 4 °C. After blocking with PBS, 2% BSA and 0.05% Tween 20 (PBS-BT), wells were preincubated for 1 h at 23 °C with PBS-BT containing no antibody, E16 IgG (50 µg/ml), E16 Fab (50 µg/ml) or E53 IgG (50 µg/ml). E53 serves as a negative control as it recognizes an epitope in domain I and II of WNV E protein. Subsequently, human plasma (1/40 dilution in PBS-BT, heat-inactivated) was directly added for an additional 1 h at 23 °C. We obtained the human samples with informed consent from seven different WNV-infected patients (gift of M. Busch and L. Tobler, Blood Systems Research Institute, San Francisco, CA). Because the samples were sequentially numbered and not linkable back to the original subjects, they satisfied the criteria for exemption from approval from the Human Studies Committee at Washington University. After six washes with PBS-BT, plates were serially incubated with biotin-conjugated goat anti-human IgG (1 µg/ml), streptavidin–horseradish peroxidase (2 µg/ml) and tetramethyl-benzidine developing substrate (DAKO). We determined optical densities at 450 nm with an automatic ELISA plate reader (Tecan) and adjusted them after subtraction of the value obtained from nonimmune human plasma.

Surface plasmon resonance. Antibody affinity analysis for DIII of WNV was performed by surface plasmon resonance (BIAcore 3000, Biacore, Inc). Binding curves and kinetic parameters were obtained as follows: we captured E16 antibodies by flowing (300 nM, rate of 5 µl/min for 2 min) them over immobilized F(ab)'2 fragment specific for goat anti-human or mouse IgG with Fc region specificity. Subsequently, DIII of the New York 1999 strain of WNV E protein (amino acids 296–415), which was generated in *E. coli*, was injected (6.25–200 nM, flow rate 70 µl/min for 1.5 min and then allowed to dissociate over 5 min). The F(ab)'2 surface was regenerated by pulse injection of 10 mM glycine (pH 1.5) and 100 mM NaOH before each E16 injection. We analyzed curves with a global fit 1:1 binding algorithm with drifting baseline.

Cloning and humanization of E16. E16 heavy- and light-chain RNA was isolated from hybridoma cells after guanidinium thiocyanate and phenol-chloroform extraction, and converted to cDNA by reverse transcription. The VH and VL segments were amplified by PCR using the 5′ rapid amplification of cDNA ends (RACE) system (Invitrogen). Gene-specific primers (GSP) for VH and VL were as follows: VH-GSP1: 5′-GGTCACTGTCACTGGCTCAGGG-3′; VH-GSP2: 5′-AGGCGGATCCAGGGGCCAGTGGATAGAC-3′; VL-GSP1: 5′-GCACACG ACTGAGGCACCTCCAGATG-3′; and VL-GSP2: 5′ CGGATCCGATGGATAC AGTTGGTGCAGCATC-3′. The PCR products were inserted into the plasmid pCR2.1-TOPO using the TopoTA kit (Invitrogen). We then subjected the resulting plasmids to DNA sequencing to determine the VH and VL sequences for E16. The cDNA sequences were translated and the predicted amino acid sequence determined. From these sequences the framework and CDR regions were identified as previously defined[42]. We joined the mouse VH to a human C-γ1 constant

region and an Ig leader sequence, and inserted it into pCI-neo for mammalian expression. We joined the mouse VL to a human Cκ segment and an Ig leader sequence and also cloned it into pCI-neo for mammalian expression of chimeric E16 (Ch-E16). For Ch-E16, site-directed mutagenesis was also performed to change residue 297 from asparagine to glutamine of the heavy chain to eliminate the single glycosylation site on the γ1 Fc.

Humanized E16 VH consists of the framework segments from the human germline VH1-18 VH segment and JH6 segment[43,44], and the CDR regions of the E16 VH, respectively. The humanized E16 VL consists of the framework segments of the human germline VK-B3 VL segment and JK2, (refs. 45–47) segment and the CDR regions of E16 VL. The humanized VH segments were assembled *de novo* from oligonucleotides and amplified by PCR. The humanized VL segments were assembled by PCR and overlapping PCR. The resulting VH and VL segments were subsequently combined by overlapping PCR with a leader sequence and the appropriate constant region segment and cloned into the expression vector pCI-neo as *Nhe*I-*Eco*RI fragments. We confirmed the DNA sequence of the resulting plasmids by sequence analysis. Site-directed mutagenesis was then performed to substitute mouse for human residues at key framework positions VH-71 (T71A) and VL-49 (Y49S). The resulting plasmids were cotransfected into human 293 cells using lipofectamine-2000 and humanized antibody was recovered from the resulting conditioned medium and purified by protein A and size-exclusion chromatography.

Statistical analysis. All data were analyzed with Prism software (GraphPad Software). For survival analysis, Kaplan-Meier survival curves were analyzed by the log-rank and Mantel-Haenszel test. For viral burden experiments, we determined statistical significance using the Mann-Whitney test.

Note: Supplementary information is available on the Nature Medicine website.

1. Granwehr, B.P. *et al.* West Nile virus: where are we now? *Lancet Infect. Dis.* **4**, 547–556 (2004).
2. Diamond, M.S., Shrestha, B., Mehlhop, E., Sitati, E. & Engle, M. Innate and adaptive immune responses determine protection against disseminated infection by West Nile Encephalitis virus. *Viral Immunol.* **16**, 259–278 (2003).
3. Wang, T. & Fikrig, E. Immunity to West Nile virus. *Curr. Opin. Immunol.* **16**, 519–523 (2004).
4. Ben-Nathan, D. *et al.* Prophylactic and therapeutic efficacy of human intravenous immunoglobulin in treating west nile virus infection in mice. *J. Infect. Dis.* **188**, 5–12 (2003).
5. Engle, M. & Diamond, M.S. Antibody prophylaxis and therapy against West Nile Virus infection in wild type and immunodeficient mice. *J. Virol.* **77**, 12941–12949 (2003).
6. Roehrig, J.T., Staudinger, L.A., Hunt, A.R., Mathews, J.H. & Blair, C.D. Antibody prophylaxis and therapy for flaviviral encephalitis infections. *Ann. NY Acad. Sci.* **951**, 286–297 (2001).
7. Modis, Y., Ogata, S., Clements, D. & Harrison, S.C. A ligand-binding pocket in the dengue virus envelope glycoprotein. *Proc. Natl. Acad. Sci. USA* **100**, 6986–6991 (2003).
8. Rey, F.A., Heinz, F.X., Mandl, C., Kunz, C. & Harrison, S.C. The envelope glycoprotein from tick-borne encephalitis virus at 2 Angstrom resolution. *Nature* **375**, 291–298 (1995).
9. Rey, F.A. Dengue virus envelope glycoprotein structure: new insight into its interactions

during viral entry. *Proc. Natl. Acad. Sci. USA* **100**, 6899–6901 (2003).

10. Modis, Y., Ogata, S., Clements, D. & Harrison, S.C. Structure of the dengue virus envelope protein after membrane fusion. *Nature* **427**, 313–319 (2004).

11. Zhang, W. *et al.* Visualization of membrane protein domains by cryo-electron microscopy of dengue virus. *Nat. Struct. Biol.* **10**, 907–912 (2003).

12. Mukhopadhyay, S., Kim, B.S., Chipman, P.R., Rossmann, M.G. & Kuhn, R.J. Structure of West Nile virus. *Science* **302**, 248 (2003).

13. Bhardwaj, S., Holbrook, M., Shope, R.E., Barrett, A.D. & Watowich, S.J. Biophysical characterization and vector-specific antagonist activity of domain III of the tick-borne flavivirus envelope protein. *J. Virol.* **75**, 4002–4007 (2001).

14. Chu, J.J. *et al.* Inhibition of West Nile virus entry by using a recombinant domain III from the envelope glycoprotein. *J. Gen. Virol.* **86**, 405–412 (2005).

15. Beasley, D.W. & Barrett, A.D. Identification of neutralizing epitopes within structural domain III of the West Nile virus envelope protein. *J. Virol.* **76**, 13097–13100 (2002).

16. Volk, D.E. *et al.* Solution structure and antibody binding studies of the envelope protein domain III from the New York strain of West Nile virus. *J. Biol. Chem.* **279**, 38755–38761 (2004).

17. Beasley, D.W. & Aaskov, J.G. Epitopes on the dengue 1 virus envelope protein recognized by neutralizing IgM monoclonal antibodies. *Virology* **279**, 447–458 (2001).

18. Crill, W.D. & Roehrig, J.T. 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**, 7769–7773 (2001).

19. Roehrig, J.T., Bolin, R.A. & Kelly, R.G. Monoclonal antibody mapping of the envelope glycoprotein of the dengue 2 virus, Jamaica. *Virology* **246**, 317–328 (1998).

20. Cecilia, D. & Gould, E.A. Nucleotide changes responsible for loss of neuroinvasiveness in Japanese encephalitis virus neutralization-resistant mutants. *Virology* **181**, 70–77 (1991).

21. Wu, S.C., Lian, W.C., Hsu, L.C. & Liau, M.Y. Japanese encephalitis virus antigenic variants with characteristic differences in neutralization resistance and mouse virulence. *Virus Res.* **51**, 173–181 (1997).

22. Lin, B., Parrish, C.R., Murray, J.M. & Wright, P.J. Localization of a neutralizing epitope on the envelope protein of dengue virus type 2. *Virology* **202**, 885–890 (1994).

23. Diamond, M.S., Shrestha, B., Marri, A., Mahan, D. & Engle, M. B cells and antibody play critical roles in the immediate defense of disseminated infection by West Nile encephalitis virus. *J. Virol.* **77**, 2578–2586 (2003).

24. Smithburn, K.C., Hughes, T.P., Burke, A.W. & Paul, J.H. A neurotropic virus isolated from the blood of a native of Uganda. *Am. J. Trop. Med. Hyg.* **20**, 471–492 (1940).

25. Boder, E.T. & Wittrup, K.D. Yeast surface display for screening combinatorial polypeptide libraries. *Nat. Biotechnol.* **15**, 553–557 (1997).

26. LoBuglio, A.F. *et al.* Mouse/human chimeric monoclonal antibody in man: kinetics and immune response. *Proc. Natl. Acad. Sci. USA* **86**, 4220–4224 (1989).

27. Meredith, R.F. *et al.* Pharmacokinetics, immune response, and biodistribution of iodine-131-labeled chimeric mouse/human IgG1,k 17-1A monoclonal antibody. *J. Nucl. Med.* **32**, 1162–1168 (1991).

28. Tao, M.H. & Morrison, S.L. Studies of aglycosylated chimeric mouse-human IgG. Role of carbohydrate in the structure and effector functions mediated by the human IgG constant region. *J. Immunol.* **143**, 2595–2601 (1989).

29. Shusta, E.V., Holler, P.D., Kieke, M.C., Kranz, D.M. & Wittrup, K.D. Directed evolution of a

30. Chambers, T.J., Halevy, M., Nestorowicz, A., Rice, C.M. & Lustig, S. West Nile virus envelope proteins: nucleotide sequence analysis of strains differing in mouse neuroinvasiveness. *J. Gen. Virol.* **79**, 2375–2380 (1998).

31. Wu, K.P. *et al.* 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**, 46007–46013 (2003).

32. Kimura-Kuroda, J. & Yasui, K. Protection of mice against Japanese encephalitis virus by passive administration with monoclonal antibodies. *J. Immunol.* **141**, 3606–3610 (1988).

33. Virgin, H.W., Bassel-Duby, R., Fields, B.N. & Tyler, K.L. Antibody protects against lethal infection with the neurally spreading reovirus type 3 (Dearing). *J. Virol.* **62**, 4594–4604 (1988).

34. Levine, B. *et al.* Antibody-mediated clearance of alphavirus infection from neurons. *Science* **254**, 856–860 (1991).

35. Diamond, M. *et al.* Modulation of dengue virus infection in human cells by alpha, beta, and gamma interferons. *J. Virol.* **74**, 4957–4966 (2000).

36. Ebel, G.D., Carricaburu, J., Young, D., Bernard, K.A. & Kramer, L.D. Genetic and phenotypic variation of West Nile virus in New York, 2002-2003. *Am. J. Trop. Med. Hyg.* **71**, 493–500 (2004).

37. Karabatsos, N. *International Catalogue of Arboviruses Including Certain Other Viruses of Vertebrates* 3rd edn (American Society of Tropical Medicine and Hygiene, San Antonio, 1985).

38. Delenda, C., Staropoli, I., Frenkiel, M.P., Cabanie, L. & Deubel, V. Analysis of C-terminally truncated dengue 2 and dengue 3 virus envelope glycoproteins: processing in insect cells and immunogenic properties in mice. *J. Gen. Virol.* **75**, 1569–1578 (1994).

39. Lanciotti, R.S. *et al.* Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. *Science* **286**, 2333–2337 (1999).

40. Harlow, E. & Lane, D. *Antibodies: A Laboratory Manual.* p. 714 (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1988).

41. Boder, E.T. & Wittrup, K.D. Optimal screening of surface-displayed polypeptide libraries. *Biotechnol. Prog.* **14**, 55–62 (1998).

42. Kabat, E.A., Wu, T.T., Perry, H., Gottesman, K. & Foeller, C. *Sequences of Proteins of Immunological Interest.* p. 3242 (US National Institutes of Health, Bethesda, Maryland, 1991).

43. Matsuda, F. *et al.* The complete nucleotide sequence of the human immunoglobulin heavy chain variable region locus. *J. Exp. Med.* **188**, 2151–2162 (1998).

44. Ravetch, J.V., Siebenlist, U., Korsmeyer, S., Waldmann, T. & Leder, P. Structure of the human immunoglobulin mu locus: characterization of embryonic and rearranged J and D genes. *Cell* **27**, 583–591 (1981).

45. Klobeck, H.G., Meindl, A., Combriato, G., Solomon, A. & Zachau, H.G. Human immunoglobulin kappa light chain genes of subgroups II and III. *Nucleic Acids Res.* **13**, 6499–6513 (1985).

46. Klobeck, H.G. *et al.* Subgroup IV of human immunoglobulin K light chains is encoded by a single germline gene. *Nucleic Acids Res.* **13**, 6515–6529 (1985).

47. Hieter, P.A., Maizel, J.V., Jr. & Leder, P. Evolution of human immunoglobulin kappa J region genes. *J. Biol. Chem.* **257**, 1516–1522 (1982).