Complexity of neutralization antibodies against multiple dengue viral serotypes after heterotypic immunization and secondary infection revealed by in-depth analysis of cross-reactive antibodies

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ABSTRACT

The four serotypes of dengue virus (DENV) cause the most important and rapidly emerging arboviral diseases in humans. The recent Phase 2b and 3 studies of a tetravalent dengue vaccine reported a moderate efficacy despite the presence of neutralizing antibodies, highlighting the need for better understanding of neutralizing antibodies in polyclonal human sera. Certain type specific (TS) antibodies were recently discovered to account for the monotypic neutralizing activity and protection after primary DENV infection. The nature of neutralizing antibodies after secondary DENV infection remains largely unknown. In this study, we examined sera from 10 vaccinees with well-documented first and second DENV serotypes exposed through heterotypic immunization of live-attenuated vaccines. Higher serum IgG avidity to both exposed and non-exposed serotypes was found after secondary immunization compared with primary immunization. Using a two-step depletion protocol to remove different anti-envelope antibodies including group-reactive (GR) and complex-reactive (CR) antibodies separately, we found GR and CR antibodies together contribute to more than 50% neutralizing activities against multiple serotypes after secondary immunization. Similar findings were demonstrated in patients after secondary infection. Anti-envelope antibodies recognizing previously exposed serotypes consist of a large proportion of GR antibodies, CR antibodies and a small proportion of TS antibodies, whereas those recognizing non-exposed serotypes consist of GR and CR antibodies. These findings have implications for sequential heterotypic immunization or primary immunization of DENV-primed individuals as alternative strategies for DENV vaccination. The complexity of neutralizing antibodies after secondary infection provides new insights into the difficulty of their application as surrogates of protection.
IMPORTANCE

The four serotypes of dengue virus (DENV) are the leading cause of arboviral diseases in humans. Despite the presence of neutralizing antibodies, a moderate efficacy was recently reported in Phase 2b and 3 trials of a dengue vaccine; a better understanding of neutralizing antibodies in polyclonal human sera is urgently needed. We studied vaccinees who received heterotypic immunization of live-attenuated vaccines as cases with known first and second DENV serotypes exposed. We found anti-envelope antibodies consist of group-reactive (GR), complex-reactive (CR) and type-specific (TS) antibodies; both GR and CR antibodies contribute significantly to multitypic neutralizing activities after secondary DENV immunization. These findings have implications for alternative strategies for DENV vaccination. Certain TS antibodies were recently discovered to contribute to the monotypic neutralizing activity and protection after primary DENV infection; our findings of the complexity of neutralizing activities after secondary immunization/infection provide new insights into neutralizing antibodies as surrogates of protection.
INTRODUCTION

Dengue virus (DENV) belongs to the Flavivirus genus of the Flaviviridae family. DENV comprises four distinct serotypes (DENV1, DENV2, DENV3 and DENV4) which circulate in tropical and subtropical regions and cause the most common and significant arboviral diseases in humans (1). It was reported recently that approximately 390 million DENV infections with 25% apparent infection occur annually, including dengue fever and the severe form of disease, dengue hemorrhagic fever and dengue shock syndrome (1-3). Despite tremendous progress in dengue vaccine development, no licensed DENV vaccine is currently available (4). Several DENV candidate vaccines have moved to clinical trials; a previous Phase 2b trial of Sanofi Pasteur’s live-attenuated chimeric yellow fever-dengue (CYD) tetravalent vaccine reported an efficacy of 30.2% (9.2% against DENV2) and recent reports of Phase 3 trials of the same vaccine revealed an efficacy of 56.5–60.8% (35.0–42.3% against DENV2), highlighting the need of a better understanding of immune responses correlated with protection (5-10).

DENV contains a positive-sense single-stranded RNA genome, which is translated into one polyprotein containing three structural proteins, capsid, precursor membrane (prM) and envelope (E) and seven non-structural proteins (11). As the major surface protein on virions, E protein participates in receptor-binding and membrane fusion and is the main target of neutralizing antibodies (Abs) (4,11). The ectodomain of E proteins contains three distinct domains. Domain I (DI) is located in the center, domain II (DII), an elongated domain containing the fusion loop (FL) at its tip, is involved in dimerization and membrane fusion, and domain III (DIII), an immunoglobulin-like domain, is involved in receptor binding and stabilization of trimers during fusion (11-14).

There are several serocomplexes in the Flavivirus genus, including the DENV serocomplex, Japanese encephalitis virus (JEV) serocomplex, tick-borne encephalitis virus serocomplex
(TBEV) and yellow fever virus (YFV). The amino acid sequence homology of E protein is about 39–49% between different serocomplexes, 63–78% between DENV serotypes, and up to 3–4% between genotypes within each DENV serotype (15). Based on the binding specificity, three categories of anti-E Abs have been identified. Anti-E Abs that recognize members of two or more serocomplexes, members within the same serocomplex, or a single member are called group-reactive (GR), complex-reactive (CR) and type-specific Abs (TS), respectively (16). Previous studies have demonstrated that different categories of anti-E monoclonal antibodies (mAbs) have different epitopes and neutralizing potency. Studies of mouse mAbs revealed that the GR mAbs primarily recognize the highly conserved residues in the FL of DII, whereas CR and TS mAbs recognize different but slightly overlapping residues in DIII (17-21). TS mAbs were generally more potent neutralizing than CR or GR mAbs (19,21). Recent studies of human mAbs reported that GR mAbs recognize either FL or both FL and bc loop residues in DII (22-24), TS mAbs recognize epitopes in DIII, interdomain residues or DI/DII hinge region (25-29), and CR mAbs recognize DIII or E dimer involving FL and other residues (25,26,30).

It is known that after primary DENV infection, individuals develop monotypic neutralizing Abs against the infecting serotype, which correlate with the life-long protection against that serotype (31-34). After secondary DENV infection, individuals develop multitypic neutralizing Abs not only against the exposed serotypes but also against the serotypes to which they have not yet been exposed (“non-exposed” serotypes) (32,35). Such heterotypic neutralizing Abs are thought to contribute to protection against the non-exposed serotypes during the third or fourth DENV infections, as suggested by the lower rates of hospital admission (36) and reduced risk of symptomatic DENV infection in humans (37) as well as lower viremia in monkeys (38-41). However, the nature of these neutralizing Abs remains elusive.

Previous studies of polyclonal human sera after DENV infection revealed that the majority of
anti-E Abs were cross-reactive, including both GR or CR Abs, whereas only a minor proportion was TS Abs (17,21,42,43). Our recent studies showed that GR anti-E mAbs derived from patients after secondary DENV infection had higher binding avidity and neutralizing potency than those from patients after primary DENV infection (23). Moreover, the concentration of GR Abs in sera correlated with the neutralizing titers against the non-exposed serotypes after secondary DENV infection (44). Based on these observations, we hypothesize that cross-reactive anti-E Abs (including GR and CR Abs) generated after secondary DENV infection become high avidity and potent neutralizing, and contribute to the neutralizing activities against the non-exposed serotypes. Since the DENV infection history is important to Abs response, we investigated vaccinees with well-documented first and second DENV serotypes exposed through heterotypic immunization of two monovalent live-attenuated vaccines (45) as well as confirmed cases of secondary DENV infection. Our in-depth analyses of anti-E Abs in polyclonal sera, including binding specificity, avidity, composition of Abs, and neutralization before and after depletion experiments, revealed that cross-reactive anti-E Abs contributed significantly to neutralizing activities against both exposed and non-exposed DENV serotypes after secondary DENV immunization/infection. This information has implications for alternative strategies of DENV vaccine development, and adds to our understanding of the complexity of neutralizing Abs after secondary DENV infection.
MATERIALS AND METHODS

Dengue-immune sera. Sera collected at day 42 post-immunization from 10 vaccinees who received heterotypic immunization of two monovalent live-attenuated DENV vaccines were studied (45). There are 4 vaccinees with immunization sequence of DENV4→DENV1, 4 with DENV4→DENV2, and 2 with DENV2→DENV1 (Table 1). Sera from three patients collected at 1 to 3 months after confirmed secondary DENV1 infection were also studied (46). Pooled serum was a mixture of convalescent-phase sera from four confirmed dengue patients as described previously (21).

Ethics statement. The 10 adult vaccinees, flavivirus-naive at baseline, were enrolled in a previously described randomized, double-blind, placebo-controlled study of heterotypic immunization at the Center for Immunization Research at the John Hopkins Bloomberg School of Public Health under an investigational new drug application approved by the US Food and Drug Administration (45,47). All study documents were approved by the Western Institutional Review Board (IRB). Informed consent was obtained in accordance with the Code of Federal Regulation (CFR 21, Part 50). The three adult dengue patients were from the Kaohsiung Medical University Hospital in Kaohsiung, Taiwan between 2002 and 2009. With the approval of the Kaohsiung Medical University IRB, informed consent was obtained. All serum samples involved in this study were coded for anonymity and their analysis was approved by the IRB of the University of Hawaii at Manoa (CHS#17568).

Western blot (WB) analysis and dot blot (DB) assay. WB analysis was performed as described previously (42). Vero cells infected with DENV1 (Hawaii), DENV2 (New Guinea C), DENV3 (H87 strain), DENV4 (H241 strain), West Nile virus (WNV, NY99 strain) or mock, were lysed in 1% NP40 lysis buffer. The cell lysates were then prepared in non-reducing sample buffer, boiled and separated in 12% polyacrylamide gel electrophoresis under 2% SDS, followed
by transfer to nitrocellulose (NC) membrane (Hybond-C Extra, GE Healthcare, UK), blocking, incubation with primary Abs (vaccinee or patient sera) and secondary Abs, and detection with enhanced chemiluminescence reagents (PerkinElmer life science, Boston, MA) (21,42). For DB assay, virus-infected Vero cell lysates were diluted in 1 X PBS and dot blotted using a 96-formatted dot-blotter (Labrepeco) to NC membrane as described previously (21).

**Virion-ELISA and IgG avidity assay.** DENV virions derived from ultracentrifugation of culture supernatants of virus-infected Vero cells or pellets of mock-infected Vero cells were UV inactivated (48), diluted in coating buffer and loaded on flat-bottom 96-well plate at 4°C overnight, followed by blocking and incubation with primary Abs (serum at 1:2000 dilution) and secondary Abs. After final wash and incubation with TMB substrate and stop solution, the optical density at 450 nm (OD450) was read with a reference wavelength of 650 nm (23,44). IgG avidity was determined by a modified ELISA with additional washing step of 8 M urea (49-51). After incubation with primary Abs (serum at 1:100 dilution) and washing, 100 μl of 8 M urea was added to each well at room temperature for 10 min, followed by washing for 4 times and incubation with secondary Abs. The avidity index equals OD450 with urea/OD450 without urea. The 8 M urea was chosen since the avidity indices based on 8 M urea correlated well with those observed with 6 M and 9 M urea (data not shown).

**Focus reduction neutralization test (FRNT).** The flat-bottom 96-well plate was seeded with Vero cells (3 X 10^4 per well) 24 h prior to infection. Two-fold serial dilutions of serum were mixed with 50 focus-forming units of DENV1 (Hawaii strain), DENV2 (NGC strain), DENV3 (CH53489), or DENV4 (H241 strain) at 37°C for 1 h; the mixtures were added to each well followed by overlay, incubation, removal of overlay, fixation and stain (17,23). The foci were counted by ImmunoSpot® reader (Cellular Technology, OH). For undepleted vaccinee serum (starting from 1:40), the FRNT_{50} titer was determined by the dilution of serum which inhibited
50% of foci compared with virus only. For patient serum and depleted serum (starting from 1:80), the FRNT50 titer was determined by a nonlinear regression analysis (GraphPad Prism 5.0).

**Depletion of cross-reactive Abs.** To deplete GR Abs, sera (1:20 dilution in 1X PBS) were incubated with NC membrane pre-coated with lysates of 293T cells transfected with prM/E expression plasmid of WNV or mock in microcentrifuge tube (0.9 ml) at room temperature for 2 h, followed by incubation with WNV virus-like particles (VLPs) or pellets derived from culture supernatants of mock-transfected 293T cells in 1X PBS at 37°C for 1 h and ultracentrifugation at 150,000×g and 4°C for 1 h to remove bound GR Abs. VLPs were prepared as described previously (21). Post-depletion sera were examined by WB analysis, DB assay, virion-ELISA and FRNT. The % reduction in FRNT50 titer by GR Abs equals \[\frac{1 – \text{FRNT}_{50} \text{ of WNV-depleted serum}}{\text{FRNT}_{50} \text{ of mock-depleted serum}}\] X 100. To further deplete CR Abs, WNV-depleted sera was incubated with NC membrane coated with cell lysates of DENV3 (a non-exposed serotype)-infected or mock-infected Vero cells, followed by incubation with UV-inactivated DENV3 virions or pellets derived from culture supernatants of mock-infected Vero cells and ultracentrifugation at 150,000×g and 4°C for 1 h to remove bound CR Abs. Compared with mock-mock-depleted sera, the % reduction in FRNT50 titer by GR and CR Abs equals \[\frac{1 – \text{FRNT}_{50} \text{ of WNV-DENV3-depleted serum}}{\text{FRNT}_{50} \text{ of mock-mock-depleted serum}}\] X 100. Compared with WNV-depleted sera, the % reduction in FRNT50 titer by CR Abs equals \[\frac{1 – \text{FRNT}_{50} \text{ of WNV-DENV3-depleted serum}}{\text{FRNT}_{50} \text{ of WNV-depleted serum}}\] X 100.

**Determination of the % GR Abs and % TS Abs.** Four-fold serial dilutions of post-depletion serum were subjected to virion-ELISA (of each of four DENV serotypes); the endpoint titers were determined by the reciprocal of the highest dilutions of post-depletion serum that reached OD450 value greater than the cut-off, 3 standard deviations of the mean OD450 value of pooled pre-immune sera of vaccinees (at 1:500 dilution) using four-parameter nonlinear
regression analysis (GraphPad Prism 5.0). After WNV-depletion, the % GR Abs equals \([1 - \frac{\text{endpoint titer of WNV-depleted serum}}{\text{endpoint titer of mock-depleted serum}}] \times 100\). After WNV-DENV3 depletion, the % TS Abs equals \([\frac{\text{endpoint titer of WNV-DENV3-depleted serum}}{\text{endpoint titer of mock-mock-depleted serum}}] \times 100\). For OD450 value (at 1:500 dilution) less than the cut-off after WNV-DENV3 depletion, the endpoint titer was <500 and the % TS Abs was estimated to be \(<500/\text{endpoint titer of mock-mock-depleted serum}\). The proportion of CR Abs (% CR Abs) was calculated by 100 – % GR Abs – % TS Abs.

**Elution of GR Abs from NC membrane.** After the depletion of GR Abs, the Abs-bound NC membranes were washed with 1 X PBS twice, followed by incubation with 0.1 M glycine-HCl (pH 2.7) at room temperature for 10 min and addition of 1 M Tris (pH 8.0) in 1/10 of total volume immediately to neutralize the acidic pH. Eluted Abs (adjusted to original volume) was tested for binding avidity by IgG avidity assay (at 1:100 dilution, the same dilution tested for serum) and epitope mapping by DB assay and capture-ELISA using WT and mutant VLPs (21).

**Statistical analysis.** The two-tailed Mann-Whitney test was used to determine the difference in avidity index, % GR Abs, % TS Abs, and reduction in FRNT50 between two groups, and two-tailed Spearman correlation test was used to determine the relationship between avidity index and FRNT50 by GraphPad Prism 5.0.
RESULTS

Binding specificity and neutralizing pattern of vaccinee sera. The basic information of the 10 vaccinees including DENV immunization serotypes, sequences and sampling time is summarized in Table 1. We first examined the binding specificity of day 42 sera from a vaccinee, who had primary DENV4 and secondary DENV2 immunizations (designated as DENV4→DENV2). WB analysis revealed detectable anti-E Abs against four DENV serotypes and West Nile virus (WNV), a member of JEV serocomplex, after primary immunization, and strong anti-E Abs with similar spectrum and moderate to weak anti-prM Abs against four DENV serotypes after secondary immunization (Fig. 1A). A similar trend was observed in vaccinees (Figs. 1C and 1E) representing the other two sequences of DENV immunization in this study (Table 1). This is in agreement with the notion that anti-E Abs are the major Abs response and GR anti-E Abs are generated after primary and secondary DENV infections (17,21,42,44). Notably, the strongest anti-E or anti-prM Abs after secondary immunization were against the primary immunization serotype in all vaccinees tested (Fig. 1 and data not shown). In addition, a monotypic neutralizing pattern against the exposed serotype after primary immunization and a multitypic neutralizing pattern against both exposed and non-exposed serotypes after secondary immunization were observed (Figs. 1B, 1D, 1F and Table 1).

GR anti-E Abs contribute to neutralizing activities against four serotypes after secondary immunization. To investigate whether GR anti-E Abs contribute to the multitypic neutralizing activities after secondary DENV immunization, we performed a depletion experiment to deplete GR anti-E Abs in two vaccinees’ sera by incubation with WNV antigen (including cell lysates containing WNV prM/E proteins and WNV VLPs), and examined the neutralizing activities by the FRNT. Compared with non-depleted and mock-depleted sera, the E-binding signals to WNV in WNV-depleted sera were undetectable or at background levels as
shown by WB analysis, DB assay and ELISA (Figs. 2A and 2C), suggesting nearly complete
depletion of GR anti-E Abs. Since anti-prM Abs after secondary DENV infection recognize prM
protein of DENV but not WNV, there is no GR anti-prM Abs (Fig. 1). Thus the Abs depleted by
WNV antigen were GR anti-E Abs (Fig. 2A).

We next examined the effect of depletion on neutralizing activities. In vaccinee ID22
(sequence of immunization: DENV4→DENV2), WNV-depleted sera showed reduction in
FRNT$_{50}$ titers to both exposed serotypes (DENV2, DENV4) and non-exposed serotype DENV1,
though less reduction to DENV3, compared to mock-depleted sera (Fig. 2B). A similar trend was
observed in another vaccinee (ID6) (Fig. 2D). The % reduction in FRNT$_{50}$ titers against four
DENV serotypes in eight vaccinees is summarized in Table 2 (p<0.0001 for each serotype,
two-tailed Mann-Whitney test), suggesting that GR anti-E Abs in polyclonal human sera
contribute significantly to the neutralizing activities against four serotypes after secondary
DENV immunization.

**Higher serum IgG avidity to exposed and non-exposed DENV serotypes after secondary
immunization compared with primary immunization.** We next investigated serum IgG avidity
by a modified virion-ELISA using 8 M urea (49-51). We included serotypes DENV1, DENV2
and DENV4 in the avidity assay since they represent both the exposed and non-exposed
serotypes of the three immunization sequences in this study (Table 1). As shown in Figs. 3D to
3F, the IgG avidity to both exposed and non-exposed serotypes, regardless of the sequences of
immunization, was significantly higher after secondary immunization compared with that after
primary immunization. Notably comparing sera after secondary immunization, the IgG avidity to
the primary immunization serotype (DENV4) was significantly higher than that to the secondary
immunization serotype (DENV2 or DENV1) in subgroups with immunization sequences of
DENV4→DENV1 and DENV4→DENV2 (Figs. 3D and 3F).
To characterize the GR Abs absorbed by WNV antigen in the depletion experiment, we eluted the GR Abs from NC membrane and examined their epitope and binding avidity. As shown in Figs. 3A and 3B, the eluted GR Abs from vaccinee ID20 showed greatly reduced binding to FL mutants (W101A plus F108A) in DB assay and VLP capture-ELISA, suggesting that the eluted GR Abs recognized these FL residues, which are two common epitope residues of human GR anti-E Abs reported previously (22-24). For vaccinees with immunization sequence of DENV4→DENV1, the avidity indices of the eluted GR Abs (ranging from 0.19 to 0.65) were higher than those of total serum after primary immunization (p=0.0002, 0.0002 and 0.0009 for DENV1, DENV2 and DENV4, respectively, two-tailed Mann-Whitney test) but lower than those of total serum after secondary immunization (Fig. 3D, p=0.0009 for each serotype, two-tailed Mann-Whitney test), suggesting that GR Abs together with other Abs such as CR or TS Abs contribute to the increased avidity of total serum after secondary immunization. A similar trend was observed for the eluted GR Abs from sera of vaccinees with the other two sequences of immunization (Figs. 3E and 3F), though the avidity indices of eluted GR Abs were significantly higher than those of total serum after primary immunization only in one or two serotypes probably due to the small sample size.

**CR anti-E Abs contribute to multitypic neutralizing activities after secondary immunization.** To investigate if CR Abs also contribute to the neutralizing activities after secondary immunization, we used DENV3 (a non-exposed serotype) antigen to additionally deplete CR Abs in WNV-depleted sera from vaccinee ID1 (DENV4→DENV1). Compared with mock-mock-depleted serum, WNV-DENV3-depleted serum showed loss of binding to DENV2 and DENV3 (the non-exposed serotypes) suggesting a complete depletion of CR Abs (Fig. 4A). The remaining binding signals to DENV4 (the primary immunization serotype) and relatively low signals to DENV1 (the secondary immunization serotype) represent the TS Abs (Figs. 4A
and 5C, serum dilution of 1:2000 and 1:500, respectively). A similar trend was observed in another vaccinee ID6 (DENV4 → DENV1) (data not shown). Analysis of the neutralizing activities in WNV-DENV3-depleted sera, compared with mock-mock-depleted serum, revealed reduction in FRNT50 titers against each serotype (Fig. 4C). The % reduction in FRNT50 titers after depleting GR and CR Abs in four vaccinees is summarized in Fig. 4D (comparing with WNV-depleted sera, p=0.0006, 0.001, 0.03 and <0.0001 for DENV1, DENV2, DENV3 and DENV4, respectively, two-tailed Mann-Whitney test). These findings suggest that CR Abs contribute to neutralizing activities against DENV after secondary immunization.

**Determining the proportion of GR Abs and TS Abs in sera.** Since our depletion experiment with WNV antigen and WNV plus DENV3 antigens resulted in nearly complete depletion of GR Abs and CR Abs, respectively, we sought to use the endpoint titers of each post-depletion serum to determine the % GR Abs and % TS Abs in sera. For vaccinee ID1 (DENV4→DENV1), the % GR Abs were 75.6%, 75.3%, 60.6% and 57% based on DENV1, DENV2, DENV3 and DENV4 virion-ELISAs, respectively; the % TS Abs to DENV1 and DENV4 were 0.7% and 4.1%, respectively (Figs. 5A to 5D). Fig. 5E summarizes the composition of anti-E Abs in four vaccinees’ sera. The % GR Abs ranged from 52.8% to 86.1%. The % TS Abs to the previously exposed serotypes ranged from 0.7% to 7.4%. Interestingly the % TS Abs to the primary immunization serotype (DENV4) was higher than that to the secondary immunization serotypes (DENV1 or DENV2) (p=0.0002, two-tailed Mann-Whitney test). By subtraction, the % CR Abs thus calculated ranged from 12.3% to 41.0%.

**GR anti-E Abs contribute to multitypic neutralizing activities in patients after secondary DENV infection.** To investigate if GR anti-E Abs contribute to neutralizing activities after natural secondary DENV infection, we examined sera from three patients 1 to 3 months after secondary DENV1 infection. Compared with mock-depleted serum, WNV-depleted serum
of patient ID32 showed loss of binding to WNV (Fig. 6A), suggesting a complete depletion of GR Abs. Furthermore, WNV-depleted serum showed reduction in FRNT<sub>50</sub> titers against four serotypes (Fig. 6B). Fig. 6D summarizes the reduction in FRNT<sub>50</sub> titers in three patients (p=0.0002, 0.0002, 0.007 and 0.01 for DENV1, DENV2, DENV3 and DENV4, respectively, two-tailed Mann-Whitney test). These findings suggest that GR Abs contribute to neutralizing activities against multiple DENV serotypes after natural secondary DENV infection.

Based on the endpoint titers of post-depletion sera in virion-ELISA, we determined the % GR Abs in these patients (data not shown). As summarized in Fig. 6E, the % GR Abs ranged from 56.0% to 98.5%, which were in the same range as those in vaccinees. Together, our findings suggest that vaccinees receiving heterotypic immunization of two monovalent live-attenuated vaccines generate predominant cross-reactive Abs with composition and contribution to neutralizing activities similar to those in patients after natural secondary DENV infection.

**Heterogeneity of neutralizing Abs after secondary immunization.** Figs. 7A to 7D summarize the proportion of different categories of anti-E Abs in four vaccinees after secondary immunization and their contribution to neutralizing activities. As a comparison, the proportion of TS anti-E Abs and its contribution to the monotypic neutralizing activity of a previously reported case of primary DENV3 infection are presented in Fig. 7E (27,43). In contrast to the scenario after primary DENV infection, where TS anti-E Abs constitute a small percentage of total anti-E Abs but account for the majority of neutralizing activities (27,43), our results revealed that GR and CR Abs predominate and together contribute to more than 50% neutralizing activities against two to four serotypes, suggesting the complexity of neutralizing Abs after secondary immunization with monovalent live-attenuated DENV vaccine.
DISCUSSION

In this study, we carried out an in-depth analysis of anti-E Abs in polyclonal sera from vaccinees and patients after secondary DENV immunization/infection and found that cross-reactive anti-E Abs, including GR and CR Abs, contributed significantly to neutralizing activities against three or four DENV serotypes. To our knowledge, this is the first report demonstrating the complexity of neutralizing Abs after secondary DENV immunization/infection (32,35). These findings, combined with our recent report of high-avidity and potent neutralizing human GR mAbs derived from patients after secondary infection (23), have implications for sequential heterotypic immunization or primary immunization of DENV-primed individuals. In light of recent discovery of certain TS Abs contributing to monotypic neutralizing activity and protection after primary DENV infection (52,53), our findings of the complexity of neutralizing Abs after secondary immunization/infection provide new insights into the difficulty of using neutralizing Abs as surrogates of protection in individuals receiving DENV vaccines (5-7).

We developed a two-step depletion protocol for polyclonal serum to remove GR anti-E Abs and CR anti-E Abs separately, and examined the effects on neutralizing activities. The observations that GR and CR Abs contributed to neutralizing activities against both non-exposed serotypes and exposed serotypes suggest that heterotypic immunization can generate heterotypic neutralizing activities through GR and CR Abs and booster homotypic neutralizing activities through GR and CR Abs as well. It is likely that during acute secondary DENV infection, memory B cells recognizing the conserved epitopes expand rapidly and generate higher avidity and cross-reactive neutralizing Abs through affinity maturation, as evidenced by higher level and rapid increase of serum avidity, cross-reactive memory B cells and plasmablasts compared with primary infection (49-51,54-56). It is worth noting that the contribution of GR or CR Abs to neutralization against each serotype varied (Table 2, Fig. 4D). Studies of human mAbs have
shown that many GR and CR mAbs do not neutralize the four DENV serotypes equally well (23-25,30); a potent DENV3 TS neutralizing human mAb (5J7) was reported to bind all four serotypes (“CR” Abs in binding) (57). Similarly, several DENV2 TS and DENV1 TS neutralizing human mAbs can bind multiple serotypes (26,29). These may explain different degrees of reduction in neutralizing activities among different serotypes following depletion. This also suggests that the neutralizing Abs against non-exposed serotypes generated through heterotypic immunization might vary both in their titer and duration. Whether the third dose of heterotypic immunization can generate stronger GR and CR Abs remains to be investigated. After depletion of GR and CR Abs, considerable amounts of neutralizing activities remain, designated as UD neutralizing Abs to the non-exposed serotypes (Fig. 7). One possibility is that during the depletion of GR Abs with WNV antigens (cell lysates followed by VLPs), some GR neutralizing Abs that bind to epitopes present only on virions are not depleted by the WNV VLPs and thus contribute greatly to the UD neutralizing Abs and some fraction of TS neutralizing Abs against the exposed serotypes as well. Another possibility is that some potent neutralizing CR Abs that bind better to mature than immature virions are not completely depleted by the DENV3 virions derived from Vero cells, where immature and partially immature virions constitute a large proportion (data not shown). These are supported by recent reports that the symmetry of TBEV VLPs is different from that of virions (58) and several CR potent neutralizing human mAbs recognize E-dimer epitopes on virions (30,59). Thirdly, the post-depletion serum was heated at 56°C for 30 min before FRNT but not heated before virion-ELISA, which may result in lower OD value (60,61); some undetected neutralizing Abs would contribute to FRNT titers. Together, these three possibilities might account for the UD neutralizing Abs.

Based on the endpoint titers in virion-ELISAs, anti-E Abs recognizing the exposed serotypes consist of GR, CR and TS Abs, whereas anti-E Abs recognizing the non-exposed serotypes
consist of GR and CR Abs (Fig. 5E). Interestingly, the % TS Abs recognizing the second exposed serotype was lower than that recognizing the first exposed serotype (Fig. 5E). It is conceivable that compared with the predominant and overwhelming memory B-cells recognizing the conserved GR and CR epitopes during secondary DENV infection (49,55,56), the proportion of B-cells recognizing the new TS epitope is relatively small, resulting in less TS Abs recognizing the secondary infection serotype. This finding suggests that sequential heterotypic immunization can generate TS Abs against the second, third and fourth serotypes, but the proportion of such TS Abs might be small.

Previous studies of serum IgG avidity to DENV commonly tested one, two, or mixed serotypes as antigen and have not yet addressed the avidity to different serotypes (49-51). In our avidity assay, we tested DENV1, DENV2 and DENV4, which represent both the exposed and non-exposed serotypes of the three immunization sequences in this study (Table 1), to examine if avidity to non-exposed and exposed serotypes differs. In agreement with previous reports (49-51), serum IgG avidity was significantly higher after secondary immunization compared with that after primary immunization (Fig. 3). Moreover, IgG avidity to each serotype correlated with the neutralizing titers against that serotype for DENV1 and DENV2 but not for DENV4 (correlation coefficient $r=0.85$, $p<0.0001$; $r=0.54$, $p=0.016$; and $r=0.38$, $p=0.12$; respectively, two-tailed Spearman correlation test) (data not shown).

Consistent with the principle of original antigenic sin described for DENV infection (62), the strongest anti-E or anti-prM Abs band after secondary immunization was against the primary immunization serotype based on WB analysis (Fig. 1 and data not shown). However, a higher neutralizing Abs titer against the primary immunization serotype was found in only 6 of 10 vaccinees (Table 1), which is in agreement with a previous report (63). This is probably because the neutralizing Abs represent only a small subset of total anti-E Abs in polyclonal sera and could
be affected by DENV strains used, whereas the majority of anti-E Abs are cross-reactive and can
be detected by DENV antigens derived from different strains in most binding assays. Consistent
with this interpretation, we found higher serum IgG avidity to the primary immunization
serotype than to the secondary immunization serotype (Figs. 3D and 3F). A recent study reported
that serum IgG avidity to the primary infection serotype was higher during the acute phase but
lower than that observed for the secondary infection serotype after 3 to 18 months, suggesting a
possible replacement of secondary serotype-specific long-lived plasma cells in the bone marrow
(64). In this regard, variation in the timing of such replacement in bone marrow may account for
the different results in our study based on day 42 sera.

There are several limitations of this study. First, the sample size was small; especially only 3
patients were analyzed. It is likely that several variables might affect the Abs response such as
the sequence of infecting serotypes, time interval between infections, host genetic differences,
etc. Second, while we showed the % GR Abs and their contribution to neutralizing activities in
patients were similar to those in vaccinees, the % CR Abs and % TS Abs and their contribution
to neutralizing activities after natural secondary DENV infection remains unknown. This is due
to lack of information on the primary infection serotype and thus the non-exposed serotypes to
choose as antigen in our depletion experiment. Future studies of larger sample size and
well-documented cases with known primary, secondary and/or tertiary infection serotypes from
prospective cohort studies are needed to further our understanding of Abs responses to DENV
natural infection. Third, we studied cross-reactive Abs from day 42 to 3 months after secondary
DENV exposure. Whether these cross-reactive Abs are present in sufficient amount during the
acute viremic stage of infection to prevent severe disease or in suboptimal concentration thus
contributing to ADE remains to be investigated. Previous studies have shown that maternal
neutralizing Abs titers correlate with the age at onset of DHF/DSS in infants (65,66), suggesting
the timing and concentration of cross-reactive Abs during DENV infection is critical for disease pathogenesis. In addition, future studies on B-cell populations will complement our analysis on the circulating Abs, as a recent study using deep sequencing showed increased expansion of B-cell clones with consistent Abs sequence features in the complementarity-determining region 3 after acute DENV infection (67).

To explain the low efficacy (overall 30.2%, DENV2 9.2%) despite the presence neutralizing Abs in Sanofi Pasteur’s Phase 2b vaccine trial, several possibilities have been proposed, including the need for detailed analysis of pre- and post-vaccination samples on an individual level for those with breakthrough infection, the lack of DENV non-structural proteins in the CYD vaccine to induce protective T-cell responses (68), the possible antigenic mismatch of circulating genotypes and vaccine strains, and the overestimation of neutralizing Abs titers using non-FcγR-bearing cell-based neutralization assays (8-10). With regards to the relevance of current neutralization assays, it is worth noting that neutralizing titers higher than 1:10 has been reliably used as a surrogate of protection in two successful human flaviviral vaccines, JEV and YFV vaccines, each targeting a single member in the Flavivirus genus (69-71). In the case of primary DENV infection, the presence of monotypic neutralizing activity correlates with life-long protection against that serotype (31-35). Such monotypic neutralizing activity can be depleted by DENV antigen of the same serotype but not by that of non-exposed serotypes, suggesting that only TS Abs, rather than cross-reactive GR or CR Abs, contribute to such monotypic neutralizing activity (27). The epitopes recognized by the TS neutralizing Abs after primary DENV3 infection were recently reported to be residues in the DI/DII hinge region, while two residues proximal to the DI/DII hinge region comprised the epitope following immunization of monovalent DENV1 live-attenuated vaccine (52,53), suggesting the importance of TS Abs recognizing such epitopes to confer protection. Our findings suggest that GR and CR Abs
contribute to the neutralizing activities against the non-exposed serotypes after secondary DENV immunization/infection and may mask the detection of such protective TS neutralizing Abs. Since about 25% and 30% of monovalent and bivalent responses, respectively, were elicited after first dose of CYD vaccine (72), it is conceivable that these individuals would experience secondary DENV infection during the second or third dose and generate neutralizing Abs to the third or fourth serotypes, of which some are contributed by GR and CR Abs rather than TS neutralizing Abs. This may explain why neutralizing Abs against all four serotypes achieved after three doses of CYD vaccine do not correlate with protection against certain serotypes. Our findings also suggest that in future assays to detect “protective” TS neutralizing Abs in polyclonal sera of vaccinees receiving tetravalent live-attenuated vaccine, GR and CR Abs may need to be depleted to avoid confounding effects.
ACKNOWLEDGMENTS

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FIGURE LEGENDS

FIG 1 Binding specificity and neutralizing activities of vaccinee sera. Day 42 sera from three vaccinees (ID 19, A and B; ID1, C and D; ID12, E and F) after primary and secondary immunization were subjected to WB analysis at 1:500 dilution (A,C,E) and FRNT (B,D,F). Previously described mouse GR anti-E mAbs (FL0232, 4G2) (21) were used to verify comparable amounts of DENV and WNV antigens were loaded (G). Data are mean and standard deviation of duplicates from one of two experiments. WN, WNV; Mo, mock.

FIG 2 Contribution of GR anti-E Abs to neutralizing activities after secondary immunization. Day 42 sera from two vaccinees (ID22, A and B; ID6, C and D) after secondary immunization were depleted with WNV antigen or mock to remove GR Abs as described in Methods. Post-depletion sera were examined by WB analysis, DB assay, virion-ELISA at 1:2000 dilution (A,C) and neutralizing titers (FRNT50) (B,D). A previously described mouse GR anti-E mAb (FL0232) (21) was used to verify comparable amounts of DENV and WNV antigens were loaded in WB analysis and DB assay (A, right). Data are mean and standard deviation of two experiments, each with duplicates. WN, WNV; Mo, mock. *p=0.03, two-tailed Mann-Whitney test, comparing OD450 values (A,C) and relative FRNT50 (B,D) between WNV- and mock-depleted sera to each serotype or WNV.

FIG 3 Epitopes and IgG avidity of eluted GR Abs after secondary immunization compared with total serum IgG avidity after primary and secondary immunization. (A,B) Day 42 serum from vaccinee ID20 after secondary DENV2 immunization was depleted with WNV antigen; GR anti-E Abs were eluted from NC membrane as described in Methods and subjected to epitope
mapping by DB assay (A) and capture-ELISA using WT and mutant VLPs containing key FL mutations (B) (21). (C–E) Avidity indices of eluted GR anti-E Abs and total serum were determined as described in Methods. Vaccinees were subgrouped by sequence of immunization including DENV4→DENV1 (ID1, ID4, ID5 and ID6 in D), DENV2→DENV1 (ID11 and ID12 in E), and DENV4→DENV2 (ID16, ID19 and ID20 in F). A pooled DENV-immune human serum was used to verify comparable amounts of DENV were loaded in the virion-ELISA (C). Data are mean and standard deviation of duplicates from one of two experiments (A to C), and mean and standard deviation of duplicates from two experiments for all individuals within each subgroup (D to E). P-value was determined by two-tailed Mann-Whitney test.

**FIG 4** Contribution of CR anti-E Abs to neutralizing activities after secondary immunization. (A–C) Day 42 sera from vaccinee ID1 after secondary immunization were depleted with WNV antigen and WNV plus DENV3 antigens to remove GR Abs and GR plus CR Abs, respectively, as described in Methods. Depletion with mock and mock-mock served as control. Post-depletion sera were examined by virion-ELISA at 1:2000 dilution (A) and FRNT (C). A human anti-E GR mAb (82.11LALA) was used to verify comparable amounts of DENV and WNV antigens were loaded (B) (25,44). Data are mean and standard deviation from two experiments, each with duplicates. *+p=0.03, two-tailed Mann-Whitney test, comparing OD450 values (A) between WNV- and mock-depleted sera (*) or between WNV-D3- and mock-mock-depleted sera (†) to each serotype or WNV, and relative FRNT50 (C) between WNV-D3- and mock-mock-depleted sera (‡) to each serotype. (D) Summary of the % reduction in FRNT50 titers after depletion with WNV plus DENV3 antigens in four vaccinees. Data are mean from two experiments, each with duplicates. P-value was determined by comparing the % reduction in FRNT50 titers (all data
points of 4 vaccinees) against each serotype between WNV-D3- and mock-mock-depleted or WNV-depleted sera, two-tailed Mann-Whitney test. WN, WNV; Mo, mock.

**FIG 5** Composition of anti-E Abs in sera after secondary immunization. (A,B) Day 42 serum from vaccinee ID1 after secondary (DENV4→DENV1) immunization was depleted with WNV antigen and examined by virion-ELISA at 1:2000 dilution (A). Four-fold serial dilutions of the WNV- and mock-depleted sera were subjected to virion-ELISA of each serotype to determine the endpoint titers and % GR Abs (B) as described in Methods. (C,D) WNV-depleted serum was further depleted with DENV3 (a non-exposed serotype) antigen and examined by virion-ELISA at 1:2000 (C, left, the same graph as Fig. 4A for comparison) and 1:500 (C, right) dilutions. Four-fold serial dilutions of the WNV-DENV3- and mock-mock-depleted sera were subjected to virion-ELISA of DENV1 and DENV4 to determine the endpoint titers and % TS Abs (D) as described in Methods. (E) Summary of the composition of anti-E Abs in sera of four vaccinees. The % CR Abs was calculated by 100 – % GR Abs – % TS Abs. * p=0.03, two-tailed Mann-Whitney test, comparing OD450 values between WNV- and mock-depleted sera (*) or between WNV-D3- and mock-mock-depleted sera (’) to each serotype or WNV, Data are mean and standard deviation of duplicates from two experiments. WN, WNV; Mo, mock.

**FIG 6** Contribution of GR anti-E Abs to neutralizing activities in patients after secondary DENV infection. (A,B) Sera from patient ID32 at 3 months after secondary DENV1 infection were depleted with WNV antigen or mock. Post-depletion sera were examined by virion-ELISA at 1:2000 dilution (A) and FRNT (B). A mouse (FL0232) and human (82.11LALA) GR anti-E mAbs were used to verify comparable amounts of DENV and WNV antigens were loaded in the virion-ELISA (25,44) (C). Data are mean and standard deviation of two or three experiments,
each with duplicates. *p=0.002, **p=0.009, *p=0.03, two-tailed Mann-Whitney test, comparing
OD450 values (*,**) (A) and relative FRNT<sub>50</sub> (*) (B) between WNV- and mock-depleted sera to
each serotype or WNV. (D) Summary of the % reduction in FRNT<sub>50</sub> titers against 4 serotypes
after depletion with WNV antigen in three patients. Data are mean from two experiments, each
with duplicates. P-value was determined by comparing the % reduction in FRNT<sub>50</sub> titers (all data
points of 3 patients) between WNV- and mock-depleted sera to each serotype, two-tailed
Mann-Whitney test. (E) Four-fold serial dilutions of WNV- and mock-depleted sera were
subjected to virion-ELISA of each serotype to determine the endpoint titers as in Fig. 5. The %
GR Abs in three patients’ sera after secondary DENV<sub>1</sub> infection is summarized. Data are mean of
duplicates from one of two experiments. WN, WNV; Mo, mock.

**FIG 7** Composition of anti-E Abs and contribution of different anti-E Abs to neutralizing
activities in four vaccinees after secondary immunization. (A–D) The proportion of different
anti-E Abs (% GR, CR and TS Abs) were based on Fig. 5E, and the contribution of GR and CR
Abs to neutralizing activities was based on the % reduction of FRNT<sub>50</sub> titers in Table 2 and Fig.
4D for four vaccinees (ID1, A; ID6, B; ID19, C; ID20, D). After depletion of GR and CR Abs,
the remaining neutralizing activities to previously non-exposed serotypes are designated as
undetermined (UD). (E) As a comparison, data from a previously reported case of primary
DENV<sub>3</sub> infection were summarized. Shown are the % TS anti-E Abs, estimated by endpoint
titers in recombinant DIII- and virion-ELISAs (43), and its contribution to the monotypic
neutralizing activity (27).
**TABLE 1** Basic information and serum neutralization titers of vaccinees in this study

<table>
<thead>
<tr>
<th>Vaccinee ID</th>
<th>Interval (years)</th>
<th>Primary and secondary infection serotype</th>
<th>Time of sampling</th>
<th>FRNT$_{50}$ titers$^{d}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>D1</td>
</tr>
<tr>
<td>1</td>
<td>1.3</td>
<td>D4, primary</td>
<td>day 42</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D1, secondary</td>
<td></td>
<td>2560</td>
</tr>
<tr>
<td>4</td>
<td>1.4</td>
<td>D4, primary</td>
<td>day 42</td>
<td>&lt;40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D1, secondary</td>
<td></td>
<td>2560</td>
</tr>
<tr>
<td>5</td>
<td>7.4</td>
<td>D4, primary</td>
<td>day 42</td>
<td>&lt;40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D1, secondary</td>
<td></td>
<td>480</td>
</tr>
<tr>
<td>6</td>
<td>1.9</td>
<td>D4, primary</td>
<td>day 42</td>
<td>60</td>
</tr>
<tr>
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<td></td>
<td>D1, secondary</td>
<td></td>
<td>640</td>
</tr>
<tr>
<td>11</td>
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<td>160</td>
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<td></td>
<td>D1, secondary</td>
<td></td>
<td>1920</td>
</tr>
<tr>
<td>12</td>
<td>2.1</td>
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<td>day 42</td>
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<td>16</td>
<td>4.0</td>
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<td>day 42</td>
<td>&lt;40</td>
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<td></td>
<td></td>
<td>D2, secondary</td>
<td></td>
<td>480</td>
</tr>
<tr>
<td>19</td>
<td>5.9</td>
<td>D4, primary</td>
<td>day 42</td>
<td>&lt;40</td>
</tr>
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<td>D2, secondary</td>
<td></td>
<td>1280</td>
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<tr>
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<td>D2, secondary</td>
<td>day 42</td>
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<td></td>
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<tr>
<td>22</td>
<td>6.6</td>
<td>D2, secondary</td>
<td>day 42</td>
<td>2560</td>
</tr>
</tbody>
</table>

$^a$Ten vaccinees receiving heterotypic immunization with live-attenuated DENV vaccine were included in this study (45).

$^b$The interval (years) between the first and second immunizations (45).

$^c$The day after the first or second immunization (45). NA: not available.

$^d$The neutralizing titers (FRNT$_{50}$) on Vero cells (23). D1: DENV1; D2: DENV2; D3: DENV3; D4: DENV4. Data are mean of duplicates from one of two experiments.
<table>
<thead>
<tr>
<th>Vaccinee ID</th>
<th>Immunization serotypes</th>
<th>Reduction in FRNT50 titers (%)&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>primary → secondary</td>
<td>D1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>D4 → D1</td>
<td>22.5</td>
</tr>
<tr>
<td>4</td>
<td>D4 → D1</td>
<td>28.8</td>
</tr>
<tr>
<td>5</td>
<td>D4 → D1</td>
<td>53.4</td>
</tr>
<tr>
<td>6</td>
<td>D4 → D1</td>
<td>50.9</td>
</tr>
<tr>
<td>16</td>
<td>D4 → D2</td>
<td>54.6</td>
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<td>D4 → D2</td>
<td>51.0</td>
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<td>20</td>
<td>D4 → D2</td>
<td>46.7</td>
</tr>
<tr>
<td>22</td>
<td>D4 → D2</td>
<td>64.0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Vaccinees received heterotypic immunization with live-attenuated DENV vaccine (45).

<sup>b</sup>The neutralizing titers (FRNT<sub>50</sub>) on Vero cells were determined (23). D1: DENV1; D2: DENV2; D3: DENV3; D4: DENV4. Day 42 sera after secondary immunization were depleted of GR anti-E Abs. The % reduction in FRNT<sub>50</sub> titers, determined as described in Methods, were presented as mean from two experiments, each with duplicates.

<sup>c</sup><sup>p</sup><0.0001, two-tailed Mann-Whitney test, comparing the % reduction in FRNT<sub>50</sub> titers between WNV-depleted and mock-depleted sera (all data points of 8 vaccinees) for each serotype.
F4

A

B

C

D

<table>
<thead>
<tr>
<th>Vaccinee ID</th>
<th>Sequence of infection</th>
<th>Depletion</th>
<th>Reduction in FRNT50 titers (%)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>D1</td>
</tr>
<tr>
<td>1</td>
<td>D4 → D1</td>
<td>WNV + D3</td>
<td>45.3</td>
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<tr>
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<td>WNV + D3</td>
<td>59.5</td>
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<td>19</td>
<td>D4 → D2</td>
<td>WNV + D3</td>
<td>62.6</td>
</tr>
<tr>
<td>20</td>
<td>D4 → D2</td>
<td>WNV + D3</td>
<td>58.3</td>
</tr>
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</table>

Compared with Mo-Mo-depleted: p<0.0001, p<0.0001, p<0.0001, p<0.0001

Compared with WN3-depleted: p=0.0006, p=0.001, p=0.03, p<0.0001
A

B

C

D

E

<table>
<thead>
<tr>
<th>Vaccinee ID</th>
<th>% GR Abs</th>
<th>% TS Abs</th>
<th>% CR Abs</th>
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<tr>
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<td>D1-ELISA D2-ELISA D3-ELISA D4-ELISA</td>
<td>D1 D2 D3 D4</td>
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<tr>
<td>1</td>
<td>75.6 75.3 60.6 57.0</td>
<td>0.7 1.5 1.6 2.5</td>
<td>23.7 – 39.4</td>
</tr>
<tr>
<td>6</td>
<td>66.5 71.5 67.9 52.8</td>
<td>1.5 6.2 4.4</td>
<td>28.5 – 41.0</td>
</tr>
<tr>
<td>19</td>
<td>85.8 86.1 82.6 72.7</td>
<td>1.6 4.4</td>
<td>12.3 – 22.9</td>
</tr>
<tr>
<td>20</td>
<td>71.0 74.1 69.8 63.2</td>
<td>2.5 7.4</td>
<td>23.4 – 30.2</td>
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* For OD450 (at 1:500 dilution) < cut-off after WNV-D3 depletion, % TS Abs was estimated to be < 0.3% as described in Methods.
### Table

<table>
<thead>
<tr>
<th>Patient ID</th>
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</tr>
<tr>
<td>22</td>
<td>97.8</td>
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</table>

### Time of sampling

- 1 month
- 3 months
- 3 months

### Reduction in FRNT50 titers (%)

<table>
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<tr>
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<th>Reduction</th>
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<tr>
<td>D3</td>
<td>62.9</td>
</tr>
<tr>
<td>D4</td>
<td>62.9</td>
</tr>
</tbody>
</table>

### OD 450 nm

- 0.5
- 1.0
- 1.5
- 2.0

### Graphs

- OD 450 nm vs. Time of sampling
- Reduction in FRNT50 titers (%) vs. OD 450 nm