

JVI00556-18 Revised

**Potent neutralizing human monoclonal antibodies preferentially target
mature dengue virus particles: implication for novel strategy of dengue
vaccine**

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- 26 Key words: dengue virus; envelope; mature particles; monoclonal antibody; neutralization
- 27 Running title: Binding of human mAbs to mature dengue viral particles
- 28 Word count for abstract: 249
- 29 Word count for text: 4,309

30 **ABSTRACT**

31 The four serotypes of dengue virus (DENV) cause the most important mosquito-borne viral
32 disease in humans. The envelope (E) protein is the major target of neutralizing antibodies and
33 contains 3 domains (DI, DII and DIII). Recent studies reported that human monoclonal
34 antibodies (mAbs) recognizing the DIII, D1/DII hinge, the E-dimer epitope or a quaternary
35 epitope involving DI/DII/DIII are more potently neutralizing compared with those recognizing
36 the fusion-loop (FL) of DII. Due to inefficient cleavage of premembrane protein, DENV
37 suspensions consist of a mixture of mature, immature and partially immature particles. We
38 investigated the neutralization and binding of 22 human mAbs to DENV1 virions with
39 differential maturation status. Compared with FL mAbs, DIII, DI/DII hinge and E-dimer epitope
40 mAbs showed higher maximum binding and avidity to mature particles relative to immature
41 particles; this feature may contribute to the strong neutralizing potency of such mAbs.
42 FL-specific mAbs required 57 to 87% occupancy on mature particles to achieve half maximal
43 neutralization (NT₅₀), whereas the potently neutralizing mAbs achieved NT₅₀ states at 20 to 38%
44 occupancy. Analysis of the mAbs repertoire and polyclonal sera from patients with primary
45 DENV1 infection supports the immunodominance of cross-reactive anti-E antibodies over
46 type-specific antibodies. After depletion with viral particles from a heterologous DENV serotype,
47 the type-specific neutralizing antibodies remained and showed binding features shared by potent
48 neutralizing mAbs. Taken together, these findings suggest that the use of homogenous mature
49 DENV particles as an immunogen may induce more potent neutralizing antibodies against
50 DENV than immature or mixed particles.

51 **IMPORTANCE**

52 With an estimated 390 million infections per year, the four serotypes of dengue virus (DENV)
53 cause the most important mosquito-borne viral disease in humans. The dengue vaccine,
54 Dengvaxia, was licensed, however its low efficacy among dengue-naïve individuals and
55 increased risk of severe dengue in children highlight the need for a better understanding of the
56 role of human antibodies in immunity against DENV. DENV suspensions contain mature,
57 immature and partially immature particles. We investigated the binding of 22 human monoclonal
58 antibodies (mAbs) to DENV envelope protein on particles with differing maturation status.
59 Compared with weakly neutralizing mAbs, potently neutralizing mAbs had higher relative
60 maximum binding and avidity to mature particles. This was supported by analysis of mAbs
61 repertoires and polyclonal sera from patients with primary DENV infection. Together, these
62 findings suggest that mature particles may be the optimal form of presenting envelope protein to
63 induce more potent neutralizing antibodies against DENV.

64

65 INTRODUCTION

66 Dengue virus (DENV) belongs to the *Flavivirus* genus of the *Flaviviridae* family. There are four
67 serotypes (DENV1, DENV2, DENV3 and DENV4) that cause the most common and significant
68 arboviral diseases in humans (1). Approximately 390 million DENV infections with 25%
69 apparent infection occur annually, including dengue fever and the severe form of disease, dengue
70 hemorrhagic fever and dengue shock syndrome (1-4). Although the live-attenuated chimeric
71 yellow fever-dengue vaccine, Dengvaxia, has been licensed in several countries, it is
72 recommended only for persons who have experienced previous DENV infection. The moderate
73 efficacy (~60%) of Dengvaxia in the presence of neutralizing antibodies during Phase 2b and 3
74 trials, lower efficacy among dengue-naïve compared with dengue-experienced individuals (~40
75 vs. ~80%), and increased risk of hospitalization and severe dengue among young vaccinated
76 children highlight the need for a better understanding of humoral responses following natural
77 DENV infection (5-9).

78 DENV contains a positive-sense single-stranded RNA genome encoding one polyprotein,
79 which is cleaved into three structural proteins, capsid, premembrane (prM) and envelope (E) and
80 seven non-structural proteins (10). E protein, present on the surface of virion, mediates virus
81 entry and is the major target of neutralizing antibodies (4,10). The ectodomain of E protein has
82 three domains. Domain I (DI) is located in the center, domain II (DII), an elongated domain
83 containing the fusion loop (FL) at its tip, is involved in dimerization and membrane fusion, and
84 domain III (DIII), an immunoglobulin-like domain, is involved in receptor binding and
85 stabilization of trimers during fusion (10-13).

86 In the *Flavivirus* genus, there are several serocomplexes including the DENV serocomplex,
87 Japanese encephalitis virus serocomplex, tick-borne encephalitis virus serocomplex and yellow
88 fever virus as a single member. Anti-E antibodies that recognize members of two or more

89 serocomplexes, members within the same serocomplex, or a single member are categorized as
90 group-reactive (GR), complex-reactive (CR) or type-specific (TS) antibodies, respectively (14).
91 Previous studies of mouse anti-E monoclonal antibodies (mAbs) revealed that different
92 categories of mAbs recognize different epitopes and have differing neutralizing potency; murine
93 GR mAbs mainly recognize the highly conserved residues in the FL of DII, whereas CR and TS
94 murine mAbs recognize different but overlapping residues in DIII (15-19). TS mAbs were
95 generally more potent neutralizing than CR or GR mAbs (17,19). Studies of human mAbs have
96 shown that GR mAbs recognize either FL or both FL and bc loop residues in DII (20-22), TS
97 mAbs recognize DIII residues, quaternary epitope, or DI/DII hinge (DI/IIh) region (23-30), and
98 CR mAbs recognize DIII, E dimer epitope 2 (EDE2) or E dimer epitope 1 (EDE1), which
99 involve FL and other residues including the N-linked glycan at residue 153 (23,24,31,32).
100 Several of these TS mAbs such as 2D22, 14c10, 5J7 and 1F4 and CR mAbs such as C8 and C10
101 neutralize potently.

102 DENV enters the cell through receptor-mediated endocytosis. The low-pH environment in
103 endosome causes a conformational change of E protein and fusion between viral and endosomal
104 membranes (10,11,33,34). DENV particles assemble in the membranes derived from the rough
105 ER, where the immature virions bud into the lumen of ER and transport through the secretory
106 pathway (10,34). Within the low pH environment of trans-Golgi, the furin or furin-like protease
107 cleaves the prM protein on immature virions into M protein and pr peptide, which is then
108 released under neutral pH in the culture medium to form mature particles (34-36). However, the
109 prM cleavage is inefficient, leading to a mixture of mature, immature and partially immature
110 DENV particles in virus suspensions produced in tissue culture (37,38). Based on cryo-EM
111 images, the proportion of mature, immature and partially immature DENV2 particles derived
112 from C6/36 insect cells were reported to be 55, 3 or 42%, respectively (38). Using anti-prM and

113 anti-E mAbs in virion-capture ELISA, the prM-containing DENV2 particles in culture
114 supernatants derived from dendritic cells, C6/36 insect cells, Vero monkey kidney cells and 293T
115 human embryonic kidney cells were 13, 54, 52 or 63%, respectively (31). The infectivity of
116 immature particles is 10,000-fold lower compared with wild-type (mixed maturity state) particles
117 (39,40).

118 Previous studies of human antibodies to DENV primarily focused on DENV particles
119 derived from culture supernatants, which are a mixture of mature, immature and partially
120 immature particles. Little is known about how antibodies recognize DENV particles with
121 different maturation status in relationship to neutralization. In this study, we investigated the
122 neutralization and binding of 22 human anti-E mAbs on DENV1 virions with differing
123 maturation states. The findings suggest that higher relative maximum binding (B_{max}) and
124 avidity of binding to mature particles may contribute to the stronger neutralizing activities of
125 potently neutralizing mAbs. Analysis of eight polyclonal sera from subjects with prior history of
126 primary DENV1 infection revealed that TS neutralizing antibodies remained after depletion of
127 cross-reactive antibodies and showed binding characteristics shared by potently neutralizing
128 mAbs. These findings have implications for rational design and testing of new dengue vaccine
129 candidates.

130 RESULTS

131 **Binding of different categories of mAbs to mature, immature or mixed phenotype**
132 **particles.** Table 1 summarizes the source, binding specificity, domain, epitope and neutralizing
133 potency of 22 human mAbs in this study. Mature, mixed or immature DENV1 particles were
134 generated from 293T-furin cells, 293T cells or 293T cells in the presence of ammonium chloride,
135 respectively, and verified by western blot (WB) analysis (Figs. 1A and 1B). The prM protein
136 content relative to that in immature particles was determined to be 0.9 or 26.7% for mature or
137 mixed particles, respectively (Fig. 1C) (31). We first examined the binding curves of different
138 categories of mAbs to mature or immature particles. While FL-specific mAbs bound mature and
139 immature particles with similar B_{max} values, they reached B_{max} for binding to immature
140 particles at lower concentrations, suggesting a higher binding avidity to immature particles (Fig.
141 1D). The binding curves of mixed particles were between those of mature and immature particles
142 (Fig. 1E). In contrast, DIII mAbs showed higher B_{max} to mature particles compared with
143 immature particles, suggesting higher epitope accessibility on mature particles (Figs. 1F, 1G).
144 Similarly, DI/IIh, EDE1 and EDE2 mAbs had higher B_{max} to mature particles than to immature
145 particles (Figs. 1H, 1J, 1L). The binding curves of mixed particles, although between those of
146 mature and immature particles, were closer to those of mature particles than to immature
147 particles, probably due to low prM content of the mixed particles (26.7%) prepared in this study
148 (Fig. 1I, 1K, 1M).

149 **Relationship between neutralizing potency and binding characteristics of mature and**
150 **immature particles.** The half maximal neutralization (NT₅₀) values of FL-specific mAbs were
151 significantly higher than those of DIII, DI/IIh and EDE mAbs together or DIII and non-DIII
152 (DI/IIh plus EDE) mAbs alone ($P < 0.0001$, 0.002, and 0.0002, respectively, two-tailed
153 Mann-Whitney test) (Fig. 2A). DI/IIh and EDE mAbs had higher B_{max} to mature particles than

154 to immature particles ($P=0.008$, two-tailed Mann-Whitney test) (Fig. 2B), suggesting that higher
155 epitope accessibility to mature than immature particles may account for their strong neutralizing
156 potency. In addition, DI/IIh and EDE mAbs had lower dissociation constant (K_d) values for
157 mature than for immature particles ($P=0.03$, two-tailed Mann-Whitney test) (Fig. 2C), suggesting
158 that higher binding avidity to mature particles also contributed to the potent neutralization. In
159 contrast, the higher binding avidity of FL-specific mAbs to immature than to mature particles
160 may account for their lower neutralizing potency.

161 Since DENV produced in cultured cells consists of a mixture of mature, immature and
162 partially immature particles, we calculated relative B_{max} (the ratio of B_{max} of mature to that of
163 immature particles) as another parameter. We found the potent neutralizing mAbs (DIII, DI/IIh
164 and EDE mAbs together) had higher relative B_{max} values than FL-specific mAbs ($P<0.0001$
165 two-tailed Mann-Whitney test) (Fig. 2D). In addition, these potent neutralizing mAbs had lower
166 relative K_d values (the ratio of K_d of mature to immature particles) than FL-specific mAbs
167 ($P=0.001$ two-tailed Mann-Whitney test) (Fig. 2E). Taken together, these findings suggest that
168 higher relative epitope accessibility and binding avidity of potent neutralizing mAbs to mature
169 particles, which are infectious particles, compared with those of immature particles, which are
170 10,000-fold less infectious, may contribute to their potent neutralizing activity (39,40).

171 We further examined the relationship between neutralizing potency, binding avidity and
172 B_{max} of all 22 mAbs (as a group) to different particles. As shown in Fig. 3A, a positive
173 correlation was found between neutralizing potency and binding avidity to mixed particles
174 (Spearman correlation coefficient $r=0.4906$, $P=0.02$). A stronger correlation was observed
175 between neutralizing potency and binding avidity to mature particles (Spearman correlation
176 coefficient $r=0.6937$, $P=0.0003$) rather than to immature particles (Figs. 3B, 3C). While the
177 neutralizing potency correlated inversely with B_{max} to immature particles, it did not correlate

178 with Bmax to mature particles (Figs. 3D, 3E), probably due to the low Bmax for mature particles
179 for some potent neutralizing mAbs (such as DI/IIh and EDE mAbs) compared with FL-specific
180 mAbs (Fig. 2B). Using the relative Bmax in the analysis, the neutralizing potency correlated with
181 the relative Bmax to mature particles and inversely with relative Bmax to immature particles
182 (Spearman correlation coefficient $r=0.7509$, $P<0.0001$ and $r=-0.7322$, $P=0.0001$, respectively;
183 Figs. 3F, 3G).

184 **Neutralization and percent occupancy of mature and mixed particles by different mAbs.**

185 As shown in Fig. 4A, the percent occupancy of a FL-specific mAb (DVG1.3) to mature and
186 mixed particles increased as the percent neutralization increased. At the concentration of NT_{50} ,
187 the percent occupancy for mature or mixed particles reached 84% or 91%, respectively. A similar
188 trend was observed for 10 FL-specific mAbs in that the percent occupancy to mature or mixed
189 particles were 57 to 87% or 75 to 94%, respectively (4 were not tested due to NT_{50} values > 2
190 $\mu\text{g/mL}$). For a DIII mAb (470.12), the percent occupancy to mature or mixed particles at NT_{50}
191 concentration was much lower, 34% or 26%, respectively (Fig. 4B). Similarly, other categories
192 of potent neutralizing mAbs, including DI/IIh mAb (1F4), EDE1 mAb (752B10) or EDE2 mAb
193 (747B8) had the percent occupancy for mature/mixed particles at NT_{50} of 21/18%, 17/14% and
194 22/19%, respectively (Figs. 4C to 4E). Taken together, the percent occupancy of three categories
195 of potent neutralizing mAbs (DIII, DI/IIh and EDE mAbs) to mature or mixed particles at NT_{50}
196 concentration were significantly lower compared with those of FL-specific mAbs ($P<0.0001$,
197 two-tailed Mann-Whitney test) (Figs. 4F, 4G).

198 **Analysis of the repertoire of anti-E mAbs derived from the same individual.** Previous
199 studies of polyclonal human sera after DENV infection revealed that a significant proportion of
200 anti-E antibodies was cross-reactive (either GR or CR), whereas a minor proportion was TS
201 (15,19-21,41-43). To better understand the binding of the anti-E antibody repertoire to immature,

202 mature or mixed particles, we examined different anti-E mAbs derived from two individuals, one
203 with primary (DVG) and another with secondary (donor 12) DENV1 infections (Table 1). The
204 five mAbs derived from DVG included four GR mAbs and one DENV1-TS mAb. As shown in
205 Fig. 5A, the TS mAb had higher binding avidity to mixed or mature particles than the four GR
206 mAbs, whereas GR mAbs had higher Bmax to immature particles. Consistent with this finding,
207 the TS mAb had stronger neutralization potency than the four GR mAbs. The seven mAbs
208 derived from donor 12 included five GR, one CR and one DENV1-TS mAbs (Fig. 5B). The TS
209 and CR mAbs showed higher binding avidity to mixed or mature particles compared with GR
210 mAbs, whereas GR mAbs showed higher Bmax to immature particles. Notably, the TS and CR
211 mAbs had stronger neutralization potency compared with the five GR mAbs.

212 **Analysis of polyclonal dengue-immune human sera.** We next investigated 12 sera from
213 subjects following primary DENV1 infection (Table 2). We used a previously described
214 depletion protocol with inactivated DENV3, a heterologous serotype, to remove cross-reactive
215 antibodies in the serum of ID26 (Fig. 6A) (41). In agreement with previous reports (25,41), the
216 DENV1 TS neutralizing activity remained in the DENV3-depleted serum (Fig. 6B). We then
217 examined the binding of mock- or DENV3-depleted serum to mature or immature DENV1
218 particles. Compared with mock-depleted serum, which bound immature particles slightly better
219 than mature particles (endpoint titer: 114408 versus 68831), the DENV3-depleted serum showed
220 greatly reduced binding to both particles (endpoint titers: 15315 and 14180) (Figs 6C-6E). The
221 relative endpoint titer (ratio of endpoint titer of mature to immature particles) increased from
222 0.60 to 0.93 (Fig. 6C,6D). Comparing the 12 mock-depleted and DENV3-depleted sera, an
223 increase in the relative endpoint titer was observed after removing cross-reactive antibodies
224 ($P=0.027$, Wilcoxon rank signed test) (Fig. 6E), suggesting that the TS neutralizing antibodies in
225 polyclonal serum bind better to mature particles compared with immature particles.

226 DISCUSSION

227 We carried out an in-depth analysis of 22 human mAbs of different categories on the
228 neutralizing potency and binding characteristics of DENV1 virions with differing maturation
229 states. Our study presents three major findings. First, compared with FL-specific mAbs the
230 potent neutralizing mAbs recognizing DIII, DI/DII hinge and EDE had higher relative B_{max} and
231 avidity for binding to mature particles, the infectious virions. Second, potent neutralizing mAbs
232 require lower occupancy on mature particles to achieve 50% neutralization, compared with
233 FL-specific mAbs. Third, analysis of the mAbs repertoires and polyclonal sera from subjects
234 with prior history of primary DENV1 infection support the immunodominance of cross-reactive
235 antibodies over TS neutralizing antibodies, which remained after depletion experiments and
236 shared the features of potent neutralizing mAbs. Altogether, our findings suggest that mature
237 DENV particles may represent an ideal immunogen to induce potent neutralizing antibodies
238 against DENV.

239 Previous studies of murine mAbs against DENV2 reported that DIII TS strongly neutralizing
240 mAbs require lower occupancy on DENV2 particles to achieve 50% neutralization compared
241 with CR weakly neutralizing mAbs (44). Similarly, studies of murine mAbs against West Nile
242 virus (WNV) have shown that DIII potent neutralizing mAbs can achieve 50% neutralization at
243 25% occupancy on WNV particles (45,46). The binding and percent occupancy to particles with
244 different maturation states were not examined in those studies. We found that different categories
245 of potentially neutralizing human mAbs (DIII, DI/II hinge, EDE1 and EDE2) shared a common
246 feature, namely requirement of low occupancy (20 to 38%) on mature particles, the infectious
247 particles, to achieve 50% neutralization, compared with FL-specific mAbs (57 to 87%). A similar
248 trend also was observed for the mixed particles (Fig. 4F). It is worth noting that since the FL
249 epitope is exposed more times on the mixed (or immature) virions, it is expected to observe a

250 lower % occupancy of FL-specific mAbs to reach 50% neutralization on mixed (or immature)
251 virions compared with a higher % occupancy to reach 50% neutralization on mature virions.
252 However, we only used mixed virions rather than mature (or immature) virions in our
253 neutralization assays. Therefore, a higher % occupancy to mixed virions compared with mature
254 virions was observed (Figs. 4F and 4G), probably due to better binding of FL-specific mAbs to
255 mixed virions than to mature virions.

256 Since 12 mAbs from our panel were derived from two donors (DVG and donor 12), we
257 were able to investigate the anti-E mAbs repertoire from the same individual. The majority of
258 anti-mAbs from the two donors were GR and only two were TS, which had stronger neutralizing
259 potency than GR mAbs (Fig. 5). Analysis of 12 polyclonal sera from patients following primary
260 DENV1 infection revealed that they bind immature particles comparably to mature particles (Fig.
261 6E). After removal of cross-reactive antibodies via depletion experiments, the proportion of TS
262 antibodies calculated by a previously reported method (41) ranged from 12 to 43% and that of
263 cross-reactive (GR and CR) antibodies ranged from 57 to 88% (Fig. 7), supporting the
264 predominance of cross-reactive antibodies following DENV infection (15,19-21,41-43).

265 Since the discovery of potent neutralizing human mAbs targeting the DI/IIIh, DIII residues,
266 quaternary epitopes on DI, DII and DIII, and EDE, several studies have attempted to identify the
267 epitopes or domains on E protein recognized by TS neutralizing antibodies in polyclonal sera
268 following primary DENV infection. These TS neutralizing antibodies may contribute to
269 long-lived protection against the infecting serotype, and if so their epitopes would represent ideal
270 targets for each components of a tetravalent dengue vaccine. Experiments using depletion
271 protocols, mutant viruses or chimeric viruses containing transplanted epitopes to examine gain or
272 loss of neutralization have shown that, despite some individual variations, TS antibodies
273 recognizing residues proximal to DI/II hinge, DIII complex epitope residues, and DI/II hinge

274 residues contribute greatly to neutralizing activity following primary DENV1 immunization,
275 primary DENV2 infection/immunization, or primary DENV4 infection/immunization,
276 respectively (47-50, reviewed in 51).

277 The observations that these potent neutralizing human mAbs do not bind recombinant E
278 protein or DIII alone and that their epitopes involve multiple residues of different domains from
279 adjacent E monomers (such as the epitopes for mAbs 14c10, 5J7 and 2D22) suggest the
280 importance of proper conformation and arrangement of E protein on the surface of virions (or
281 virus-like particles [VLP]) to induce such potent neutralizing antibodies (25-32). Recent studies
282 have shown that stabilized E dimers can be recognized by some of these potent neutralization
283 mAbs (including mAbs 2D22, C8 and C10) (52,53) and could represent components of new
284 subunit vaccine candidates. The findings here showing that these potent neutralizing mAbs
285 recognize mature better than immature particles suggest that mature particles may represent a
286 promising immunogen to induce potent neutralizing antibodies against DENV. It is worth noting
287 that mature particles contain little pr protein compared with mixed particles and are less likely to
288 induce anti-prM antibodies, which are cross-reactive, weakly neutralizing and contribute to
289 antibody-dependent enhancement *in vitro* (31,40,56,57).

290 There are several limitations of this study. First, we focused on DENV1 particles with
291 differing maturation states and their interactions with different anti-E mAbs that were derived
292 from subjects with prior history of primary or secondary DENV1 infections as well as polyclonal
293 sera from subjects following primary DENV1 infection. Whether the information is applicable to
294 other DENV serotypes remains to be investigated in the future. Second, the DENV particles with
295 differing maturation states all were derived from human 293T cultured cells. Future studies are
296 needed to determine if DENV particles produced from other cell lines (including those from
297 mosquito origin), primary cells or *in vivo* have similar properties. Third, future cryo-EM studies

298 on other categories of human anti-E and anti-prM mAbs on DENV particles with differing
299 maturation states might provide new insights to further our understanding of the interactions
300 between human antibodies and DENV. These studies will facilitate a better understanding of
301 mechanisms of protection and enhancement, and point the way towards new strategies for DENV
302 vaccination.

303 MATERIALS AND METHODS

304 **Dengue-immune sera and ethics statement.** Serum samples from eight RT-PCR confirmed
305 DENV1 cases collected at 3 to 15 months post-infection were included in this study (Table 2).
306 The subjects were adult dengue cases with primary DENV1 infection based on monotypic
307 neutralization pattern (41); four were from the Kaohsiung Medical University Hospital in
308 Kaohsiung, Taiwan in 2006, and four from the Hilo Medical Center in Big Island, Hawaii during
309 the 2015-2016 outbreak. With the approval of the IRB of Kaohsiung Medical University and
310 University of Hawaii at Manoa (CHS#23786 and CHS#17568), written informed consent was
311 obtained. All serum samples involved in this study were coded for anonymity.

312 **WB analysis.** WB analysis was performed as described previously (41,42). Mature, mixed or
313 immature DENV1 virions purified from sucrose-gradient ultracentrifugation were prepared in
314 non-reducing sample buffer and subjected to 12% polyacrylamide gel electrophoresis, followed
315 by transfer to nitrocellulose membrane (Hybond-C Extra, GE Healthcare), hybridization with
316 dengue-immune serum plus rabbit serum against M-peptide (residues 6-27, Pacific Immunology)
317 (54) and secondary antibody (IRDye® 800CW-conjugated goat anti-human IgG at 1:10000). The
318 signal was detected by Li Cor Odyssey classic (LiCor Biosciences) and analyzed by Image
319 Studio software (41).

320 **Preparation of mature, immature and mixed DENV1 virions.** To generate immature,
321 mixed, or mature particles, DENV1 was inoculated onto 293T cells in the presence or absence of
322 10 mM ammonium chloride, and onto 293T-furin cells (a stable clone expressing furin) at
323 multiplicity of infection of 0.05, respectively. Culture supernatants collected at day 7 were
324 concentrated by Amicon column (ultra15, Amicon), and purified by 15-60% sucrose gradient
325 ultracentrifugation (SW41 at 17600 rpm, Beckman) at 4°C for 18 h, followed by buffer exchange
326 with 1X PBS.

327 **Virion-capture ELISA.** Flat-bottom 96-well plates were coated with a murine DIII mAb
328 (FL0251), which bind mature, immature or mixed particles similarly well, at 4°C overnight,
329 followed by blocking with 1% BSA in 1X PBS for 1h and addition of purified and
330 UV-inactivated mature, immature or mixed DENV1 particles, anti-E mAb (at different
331 concentrations) or a positive control serum, and anti-mouse IgG conjugated with HRP each at
332 37°C for 1 h, TMB substrate and stop solution at final step (21). The optical density (OD) at
333 wavelength of 450 nm with reference wavelength of 650 nm was read, and relative OD (rOD)
334 was normalized by the OD of the positive control serum (#17) in the same ELISA, which bound
335 mature, immature or mixed particles similarly well. Comparative amounts of different particles
336 were determined based on the OD of serum #17 (around 1.0) during the titration prior to ELISA.
337 Binding curves, Bmax, % Bmax and Kd were determined by a nonlinear regression analysis
338 (GraphPad Prism 6.0) (21). Relative Bmax and relative Kd, the ratio of Bmax and Kd of mature
339 particles to those of immature particles, respectively, were calculated. To determine the prM
340 content in particles, virion-capture ELISA was performed for mature, immature or mixed DENV1
341 particles and probed with a human anti-prM mAb (DVB59.3) or anti-E mAb (DVG17.12). The
342 relative prM content of mature and mixed DENV1 particles was determined by the ratio of OD
343 detected by anti-prM mAb to that by anti-E mAb normalized by the OD ratio of immature DENV1
344 particles (31).

345 **Virion-ELISA.** DENV virions (DENV1 to DENV4) or WNV VLP derived from
346 ultracentrifugation of culture supernatants of virus-infected/mock-infected Vero cells or WNV
347 prM/E plasmid-transfected 293T cells were UV inactivated (for virions), diluted in coating buffer
348 and coated on flat-bottom 96-well plates at 4°C overnight, followed by blocking and incubation
349 with primary (serum at 1:500 dilution) and secondary antibodies (41). After a final wash and
350 incubation with TMB substrate and stop solution, the OD at 450 nm was read with a reference

351 wavelength of 650 nm (41-43).

352 **Depletion of cross-reactive antibodies.** To deplete cross-reactive (GR or CR) antibodies,
353 sera (1:20 dilution in 1X PBS) were incubated with UV-inactivated DENV3 (from Vero cells) or
354 pellets derived from culture supernatants of mock-infected Vero cells in 1X PBS at 37°C for 1 h
355 and ultracentrifugation at 150,000×g and 4°C for 1 h to remove bound cross-reactive antibodies
356 (41). Mock- or DENV3-depleted sera were tested for neutralization and virion-ELISAs to verify
357 the depletion. Four-fold serial dilutions (starting from 1:1000) of mock- or DENV3-depleted sera
358 also were tested with DENV1 virion-ELISA (mature or immature particles) to determine the
359 endpoint titer, which was the reciprocal of the highest dilutions of post-depletion serum that
360 reached OD value greater than the cut-off (mean OD value plus 3 standard deviations of the
361 mean of dengue-naïve sera at 1:1000 dilution) using four-parameter nonlinear regression analysis
362 (GraphPad Prism 6.0). The proportion (%) of TS antibodies = endpoint titer of DENV3-depleted
363 serum/that of mock-depleted serum X100. The % cross-reactive (GR+CR) antibodies equals 100
364 - %TS.

365 **Epitope mapping.** Epitopes of human mAbs were determined by a dot blot assay using
366 lysates derived from 293T cells transfected with wild type DENV1 prM/E construct or each of
367 the 67 surface-exposed E alanine mutants, followed by verification with capture ELISA using
368 WT or mutant VLP as described previously (19,21).

369 **Microneutralization test.** Flat-bottom 96-well plates were seeded with Vero cells (3×10^4
370 cells per well) 24 h prior to infection. Two-fold serial dilutions of serum were mixed with 50
371 focus-forming units of DENV1 (Hawaii strain), DENV2 (NGC strain), DENV3 (CH53489), or
372 DENV4 (H241 strain) at 37°C for 1 h. The mixtures were added to each well followed by
373 incubation for 48-70 h, removal of medium, and fixation as described previously (21,41). After
374 adding murine mAb 4G2 and secondary antibody mixture (IRDye® 800CW-conjugated goat

375 anti-mouse IgG at 1:10000 and DRAQ5TM Fluorescent Probe at 1:10000), the signal (800
376 nm/700 nm fluorescence) was detected by Li Cor Odyssey classic (LiCor Biosciences) and
377 analyzed by Image Studio software to determine percent neutralization at different
378 concentrations and NT₅₀ (21,55).

379 **Percentage occupancy of mature and mixed particles.** Based on the binding curve of each
380 mAb, the Bmax was determined by a nonlinear regression analysis, and % Bmax, which
381 represents the percent occupancy on mature or mixed particles at different concentrations was
382 determined. Based on the neutralization curve of each mAb, the % NT was plotted against the
383 percent occupancy on mature or mixed particles, and the percent occupancy at NT₅₀
384 concentration was calculated.

385 **Statistical analysis.** Neutralizing potency and binding avidity were assessed by 1/NT₅₀ and
386 1/Kd, respectively. The two-tailed Mann-Whitney test was used to determine the difference in
387 Kd, Bmax, or NT₅₀ between two groups, and two-tailed Spearman correlation test was used to
388 determine the relationship between neutralizing potency and avidity or Bmax by GraphPad Prism
389 6.0. The two-tailed Wilcoxon rank signed test was used to determine the difference in endpoint
390 titer and relative endpoint titer between two groups (mock- and DENV3-depletion) (GraphPad
391 Prism 6.0).

392 **ACKNOWLEDGMENTS**

393 We thank Dr. Sallusto at the Institute for Research in Biomedicine, Università Della Svizzera
394 Italiana, Switzerland for kindly providing 7 human mAbs. This work was supported by awards
395 R01AI110769-01 (WKW) from the National Institute of Allergy and Infectious Diseases and
396 P30GM114737 from the National Institute of General Medical Sciences, NIH; grants
397 MOHW107-TDU-B-212-123006 (JJT) from the Ministry of Health and Welfare and
398 NHRI-MR-107-PP-38 (JJT) from the National Health Research Institute, Taiwan; grants
399 095541/Z/11/Z and 203224/Z/16/Z (GS) from The Wellcome Trust and MR/N012658/1 (GS)
400 from the Newton-Medical Research Council, and the National Institute for Health Research
401 Oxford Biomedical Research Centre, UK. The funders had no role in study design, data collection
402 and analysis, decision to publish, or preparation of the manuscript.

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596 **FIGURE LEGENDS**

597 **FIG 1** Binding of different categories of human anti-E mAbs to mature, immature or mixed
598 particles. (A) Outline of preparation of mature, mixed or immature DENV1 (D1) virions from
599 293T-furin cells, 293T cells and 293T cells in the presence of ammonium chloride, respectively.
600 (B) WB analysis of mature, mixed or immature DENV1 virions purified from sucrose-gradient
601 ultracentrifugation probed with DENV-immune human serum (anti-prM/E) and rabbit serum
602 against M-peptide (anti-M). (C) Relative prM contents of different particles were determined by
603 a virion-ELISA, which used a murine mAb (FL0251) for capture and human mAbs (anti-E:
604 DVG17.12 and anti-prM: DVB 59.3) for detection. (D-M) Binding curves of different categories
605 of human mAbs to mature, immature or mixed particles based on virion-capture ELISAs,
606 including FL-specific mAbs (D,E), DIII mAbs (F,G), EDE1 mAbs (H,I), EDE2 mAbs (J,K) and
607 DI/IIh mAb (L,M). Relative OD (rOD) to a DENV-immune human serum was presented. Data
608 are mean and standard deviation of duplicates from one representative experiment of two.

609
610 **FIG 2** Comparison of neutralizing potency, maximum binding and dissociation constant of
611 different categories of human anti-E mAbs to mature, immature or mixed particles. (A) DIII,
612 EDE and DI/IIh mAbs were more potent neutralizing than FL-specific mAbs. (B,C) Comparison
613 of the Bmax and Kd for mature or immature DENV1 particles within each category of mAbs
614 including FL, DIII, and EDE plus DI/IIh mAbs. (D,E) Comparison of relative Bmax and relative
615 Kd to mature versus immature DENV1 particles, Bmax (m/imm) and Kd (m/imm), between FL,
616 DIII, and EDE plus DI/IIh mAbs. Bmax and Kd were determined by a nonlinear regression
617 analysis (GraphPad Prism 6.0). Data are mean of duplicates from two experiments. *P* values
618 were based on two-tailed Mann-Whitney test.

619

620 **FIG 3** Relationship between neutralizing potency and binding parameters to mature, mixed or
621 immature particles. (A) Relationship between neutralizing potency and binding avidity to mixed
622 DENV1 particles. (B, D, F) Relationship between neutralizing potency and binding avidity,
623 Bmax to mature DENV1 particles and relative Bmax (mature/immature). (C, E, G) Relationship
624 between neutralizing potency and binding avidity, Bmax to immature DENV1 particles and
625 relative Bmax (immature/mature). Neutralizing potency and binding avidity were defined by
626 $1/NT_{50}$ and $1/Kd$, respectively. Bmax and Kd were determined by a nonlinear regression analysis
627 (GraphPad Prism 6.0). Data are mean of duplicates from two experiments. Spearman correlation
628 test was performed.

629
630 **FIG 4** Relationship between neutralization and percent occupancy of mature and mixed particles
631 for different categories of anti-E mAbs. (A-E) Representative example of FL (A), DIII (B),
632 DI/IIIh (C), EDE1 (D) and EDE1 (E) mAbs and percent occupancy of these mAb at NT_{50}
633 concentration. (F,G) Comparison of percent occupancy to mixed (F) or mature (G) particles at
634 NT_{50} concentration between FL mAbs and potent neutralizing mAbs (including DIII, DI/IIIh and
635 EDE mAbs). Curves were determined by a nonlinear regression analysis (GraphPad Prism 6.0).
636 Data are mean and/or standard deviation (for A to E) of duplicates from two experiments. *P*
637 values were based on two-tailed Mann-Whitney test.

638
639 **FIG 5** Analysis of repertoire of anti-E mAbs derived from the same individual. (A) The five
640 mAbs derived from a case with primary DENV1 infection (DVG) include four GR mAbs and
641 one DENV1-TS mAb. (B) The seven mAbs derived a case with secondary DENV1 infection
642 (donor 12) include five GR, one CR and one DENV1-TS mAbs. The binding curves, Kd and
643 Bmax for mature, mixed or immature particles as well as NT_{50} are presented. GR:

644 group-reactive, CR: complex-reactive, TS: type-specific. Data are mean and/or standard
645 deviation of duplicates from one representative experiment of two.

646
647 **FIG 6** Analysis of polyclonal human sera. (A) Serum from a case with prior primary DENV1
648 infection (ID26) was depleted with mock or DENV3 and tested with virion-ELISA. (B) The
649 neutralization curve for DENV1 of mock-depleted or DENV3-depleted sera from ID26. (C,D)
650 The binding curves of mature or immature DENV1 particles for mock-depleted (C) or
651 DENV3-depleted (D) sera. The endpoint titers and EC_{50} for binding to mature or immature
652 particles and the ratio (m/imm) were determined based on the four-parameter nonlinear
653 regression analysis (GraphPad Prism 6.0). (E) The endpoint titers for mature or immature
654 particles and relative endpoint titers (m/imm) of mock-depleted or DENV3-depleted sera from
655 12 patients following primary DENV1 infection. Data are mean and/or standard deviation of
656 duplicates from two experiments. Two-tailed Wilcoxon rank signed test was used.

657
658 **FIG 7** Determination of the proportion of TS antibodies in polyclonal sera. (A,B) Serum from a
659 case with prior primary DENV1 infection (ID33) was depleted with mock or DENV3 and titrated
660 on mature (A) or immature (B) DENV1 particles. The endpoint titers of mock-depleted or
661 DENV3-depleted sera were determined based on the four-parameter nonlinear regression
662 analysis (GraphPad Prism 6.0). The proportion (%) of TS antibodies=endpoint titer of
663 DENV3-depleted serum/that of mock-depleted serum X100. The % GR+CR antibodies equals
664 $100 - \%TS$. (C,D) Comparison of the %TS and % GR+CR antibodies in sera from 8 patients
665 following primary DENV1 infection based on mature (C) and immature particles (D). Data are
666 mean of duplicates from two experiments. The two-tailed Mann-Whitney test was used.

TABLE 1 The origin, binding specificity, epitope and neutralizing potency of human monoclonal antibodies in this study

mAb	Patient ID	Infection and serotype ^a	Category	Binding specificity ^b	Domain ^c	Epitopes ^d	NT ₅₀ ^e D1	Reference
DVG1.3	DVG	Primary D1	FL	GR	I/II	L107	0.52	21
DVG6.3	DVG	Primary D1	FL	GR	I/II	W101, F108	>2	21
DVG7.5	DVG	Primary D1	FL	GR	I/II	W101, F108	>2	21
DVG12.2	DVG	Primary D1	FL	GR	I/II	L107, F108, T76	>2	21
DVG17.12	DVG	Primary D1	III	TS D1	III	G383, E384, K385	0.005	21
DV470.12	donor 12	Secondary D1	III	TS D1	III	G383, E384, K385, 79E	0.01	23
DV87.1	donor 12	Secondary D1	III	CR D1-3	III	K307, E311, L389, W391	0.007	23
DV28.1	donor 12	Secondary D1	FL	GR	I/II	W101, F108	0.37	23
DV143.6	donor 12	Secondary D1	FL	GR	I/II	L107, D290, (67)	0.50	23
DV291.3	donor 12	Secondary D1	FL	GR	I/II	W101, F108	0.55	23
DV291.7	donor 12	Secondary D1	FL	GR	I/II	101W, (D290)	0.92	23
DV378.12	donor 12	Secondary D1	FL	GR	I/II	W101, F108, (D290)	0.87	23
749B6	749	Secondary D1	FL	GR	I/II	W101, F108	>2	21,31
751B6	751	Secondary D1	FL	GR	I/II	W101, F108, G78	0.53	21,31
751B11	751	Secondary D1	FL	GR	I/II	W101, F108, G106	0.65	21,31
753C1	753	Secondary D1	FL	GR	I/II	W101, F108, L107	0.14	21,31
4L5	donor 1	Secondary	FL	GR	I/II	W101, 79E, 86S	0.18	27
752B10	752	Primary	EDE1	CR	I/II	E49, Q77, W101, N134, I161, A162, P169, T200, Q323, W391, F392	0.061	31
752-2C8	752	Primary	EDE1	CR	I/II	E49, Q77, W101, I161, A162, T200, Q323, W391, F392	0.131	31
747B8	747	Secondary D2	EDE2	CR	I/II	E49, Q77, W101, N134, N153, T155, I161, A162, P169, T200, E203, Q323, W391, F392	0.030	31
747D8	747	Secondary D2	EDE2	CR	I/II	E49, Q77, W101, N134, N153, T155, I161, A162, P169, T200, K202, E203, L308, K310, Q323, W391, F392	0.021	31
1F4	donor 1	Secondary	I/II hinge	TS	I/II	K47, N52, K136, E157, T160, T161, T163, G274	0.11	27

^a Primary or secondary DENV infection and the infecting serotype of patients were determined as reported previously. D1: DENV1, D2: DENV2.

^b GR: group-reactive, CR: complex-reactive, TS: type-specific.

^c Domain was based on the location of the epitope residues or the binding to recombinant DI/II or DIII reported previously.

^d Epitope residues were reported previously or identified in this study (DV470.1, 87.1, 28.1, 143.6, 291.3, 291.7, 378.12).

^e NT₅₀ values (μg/ml) to DENV1 (D1).

TABLE 2. Basic information of DENV-immune subjects in this study

Patient ID	Type of infection ^a	Serotype ^a	Time of sampling ^b	Year and location
ID4	primary	D1	15 months	2006, Kaohsiung, Taiwan
ID7	primary	D1	15 months	2006, Kaohsiung, Taiwan
ID26	primary	D1	3 months	2006, Kaohsiung, Taiwan
ID33	primary	D1	3 months	2006, Kaohsiung, Taiwan
H1001	primary	D1	3 months	2015, Hawaii, US
H1003	primary	D1	4.5 months	2015, Hawaii, US
H1004	primary	D1	4.5 months	2015, Hawaii, US
K1002	primary	D1	5 months	2015, Hawaii, US
K1014	primary	D1	8.5 months	2015, Hawaii, US
K1015	primary	D1	8.5 months	2015, Hawaii, US
K1017	primary	D1	6.5 months	2015, Hawaii, US
K1018	primary	D1	6.5 months	2015, Hawaii, US

^a Primary or secondary DENV infection and the infecting serotype of patients were determined as reported previously (41).
^b Sampling time post-symptom onset.

Fig1

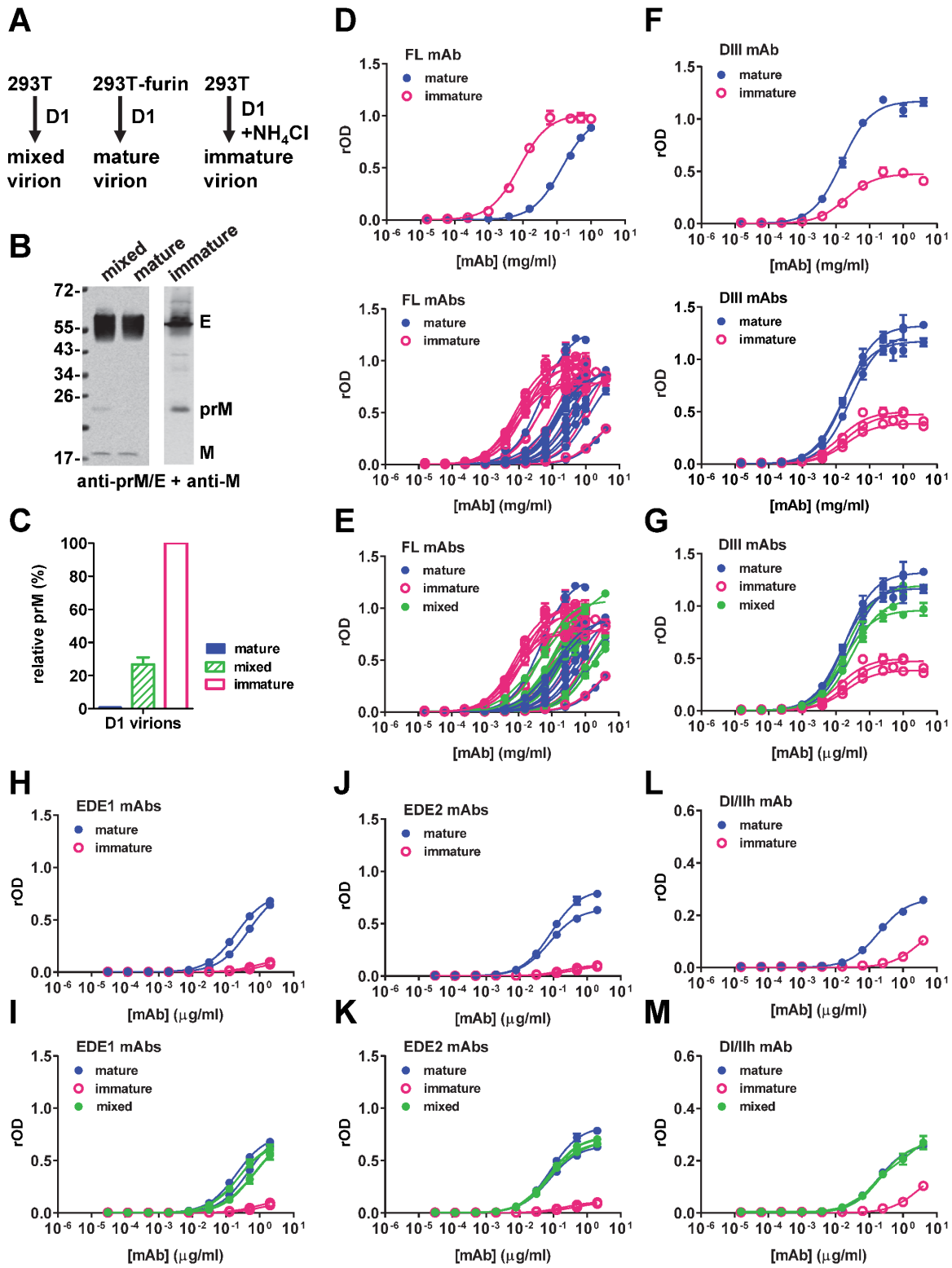


Fig2 revised

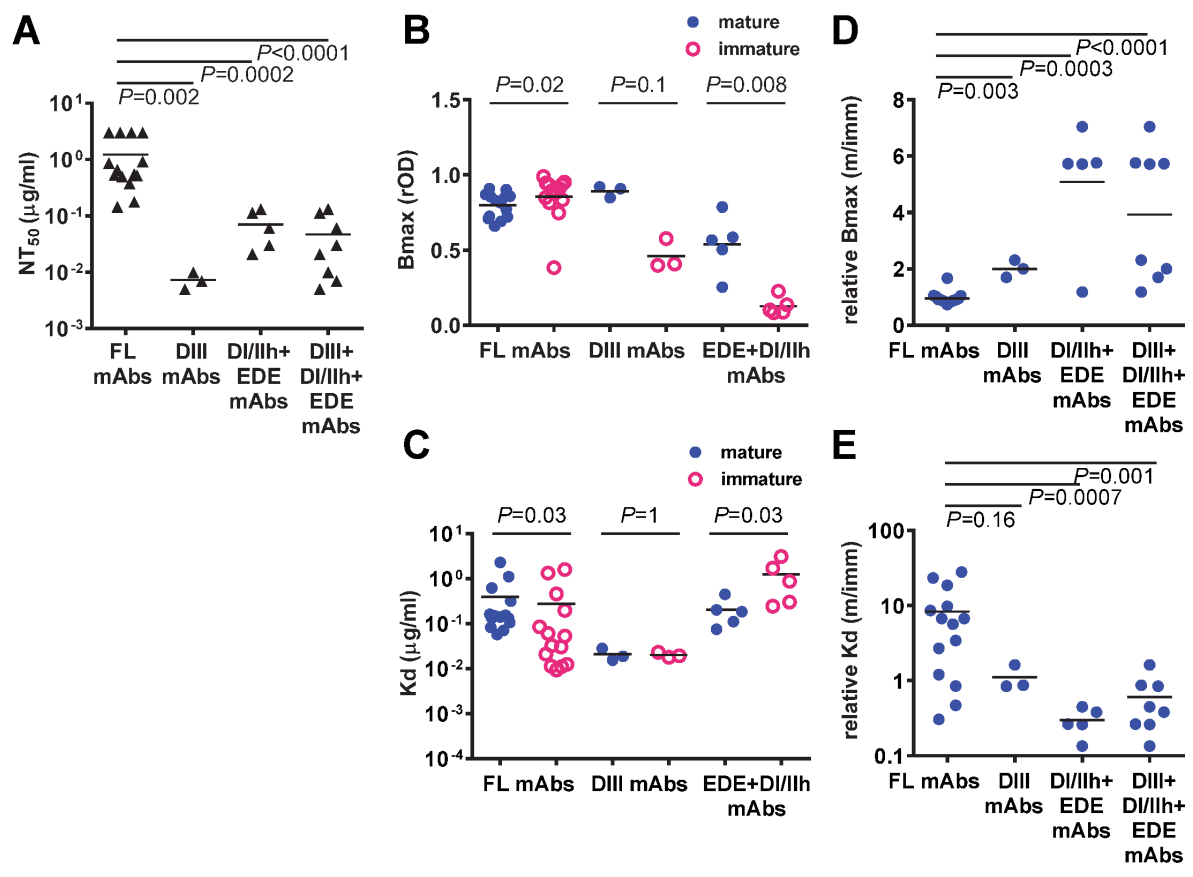


Fig3 revised

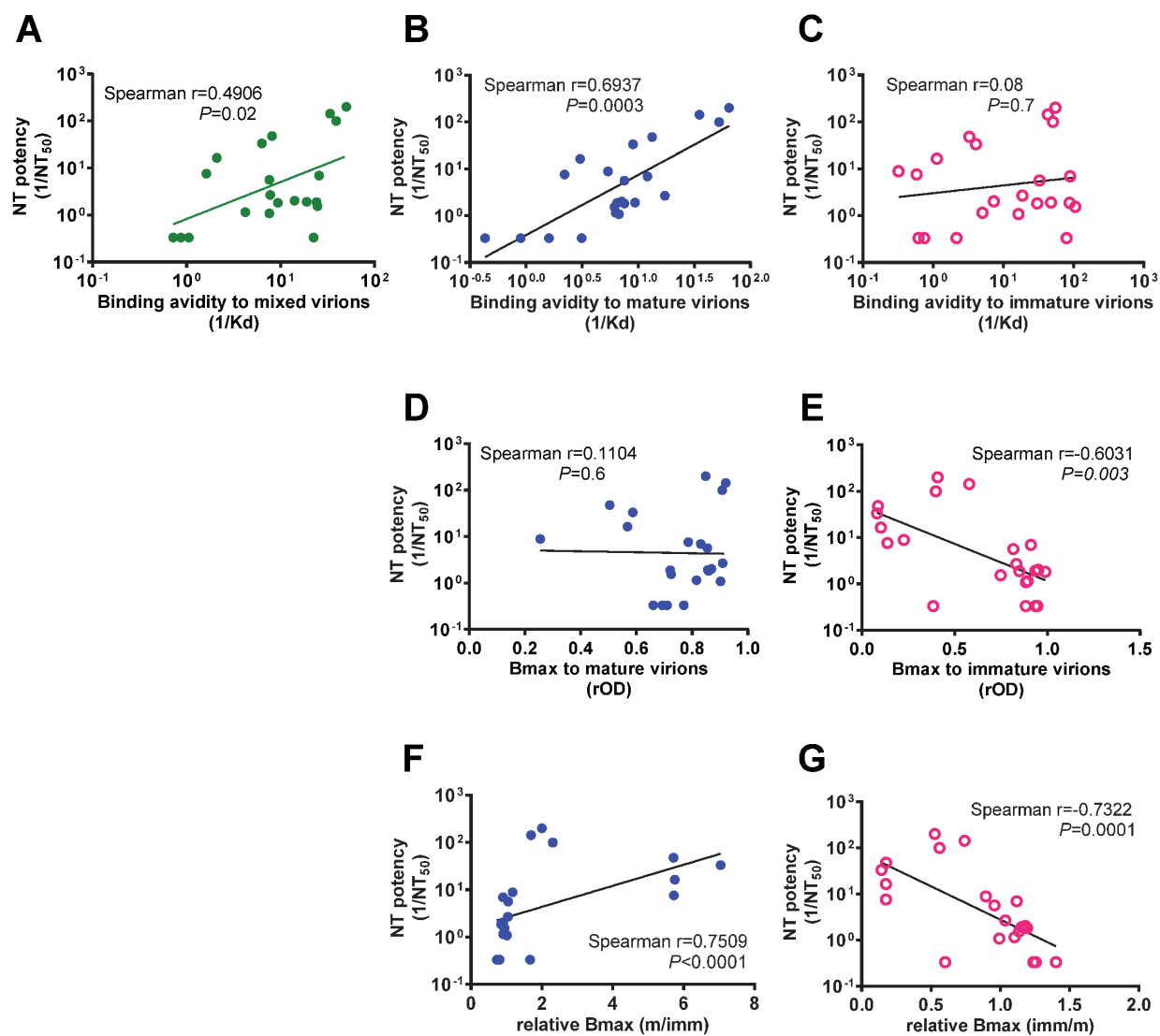


Fig4 revised

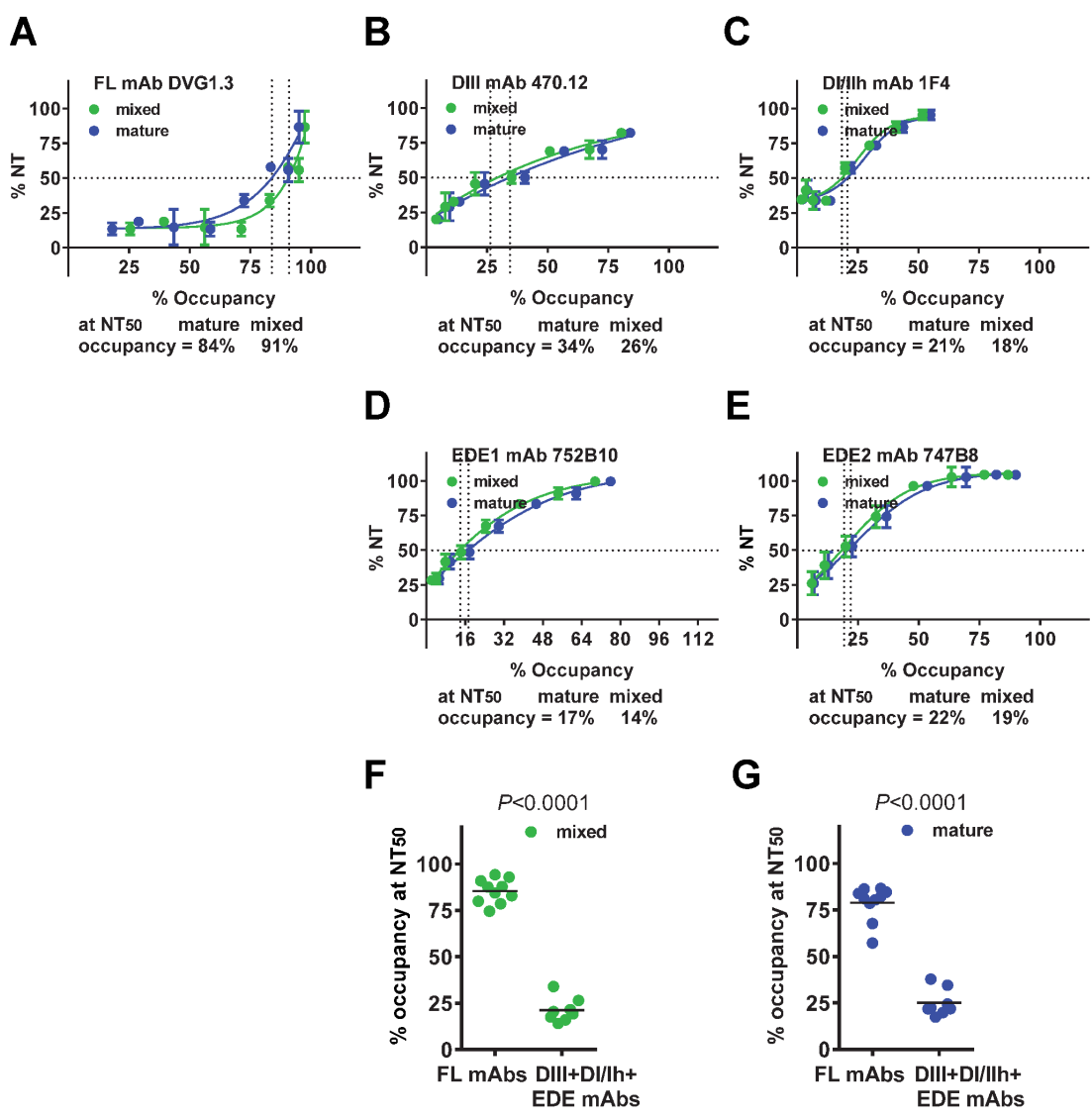
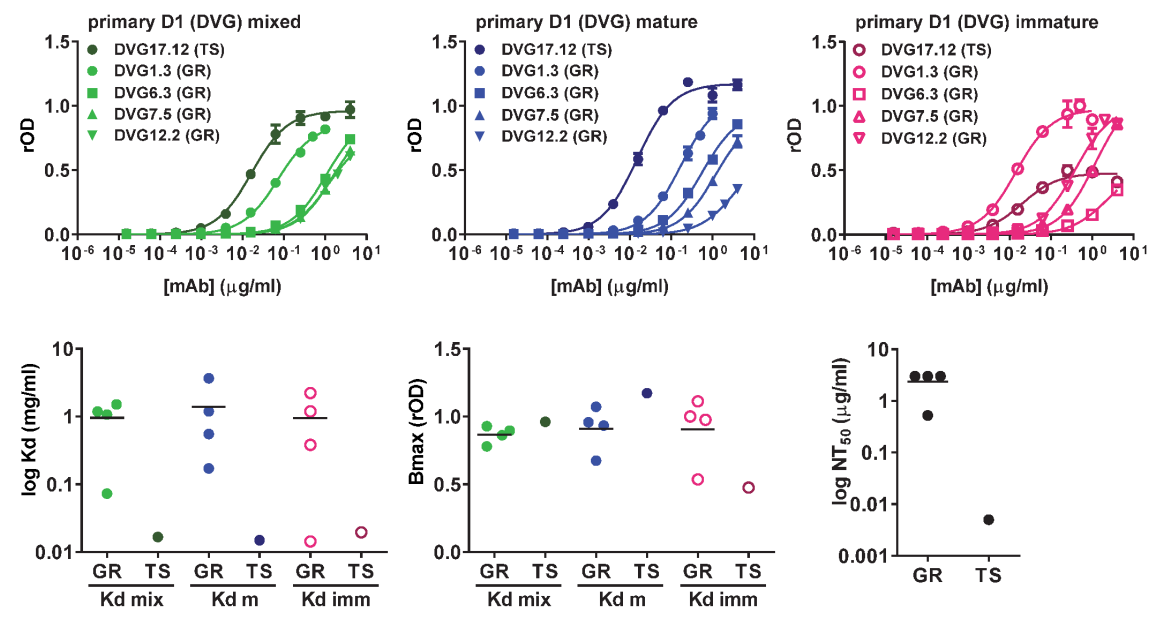


Fig5

A



B

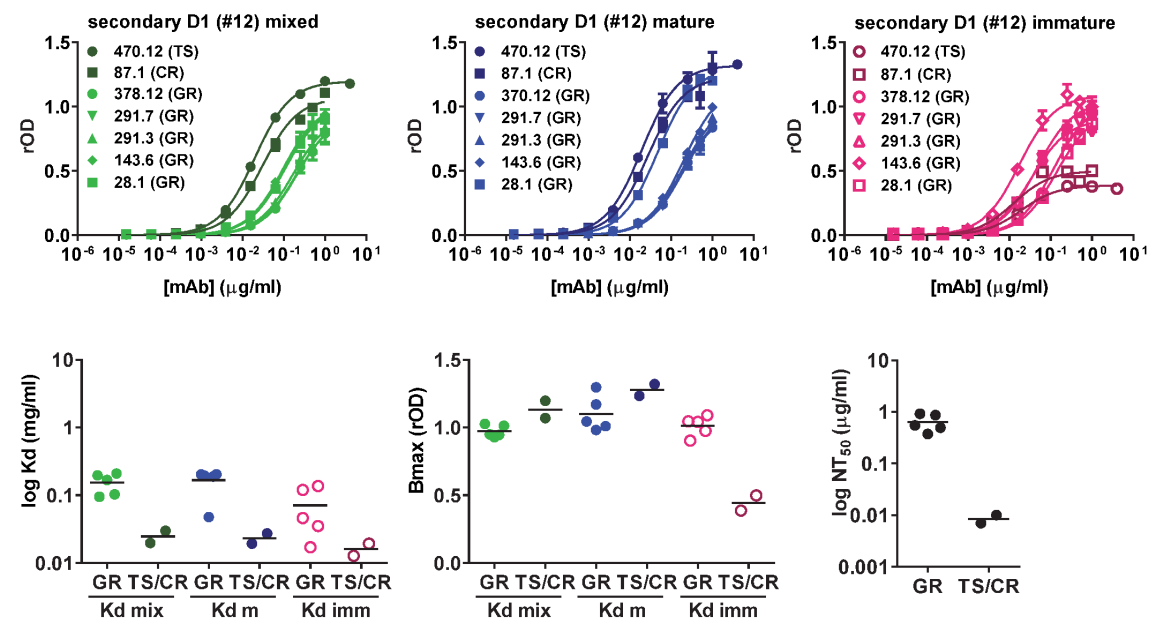


Fig6 revised

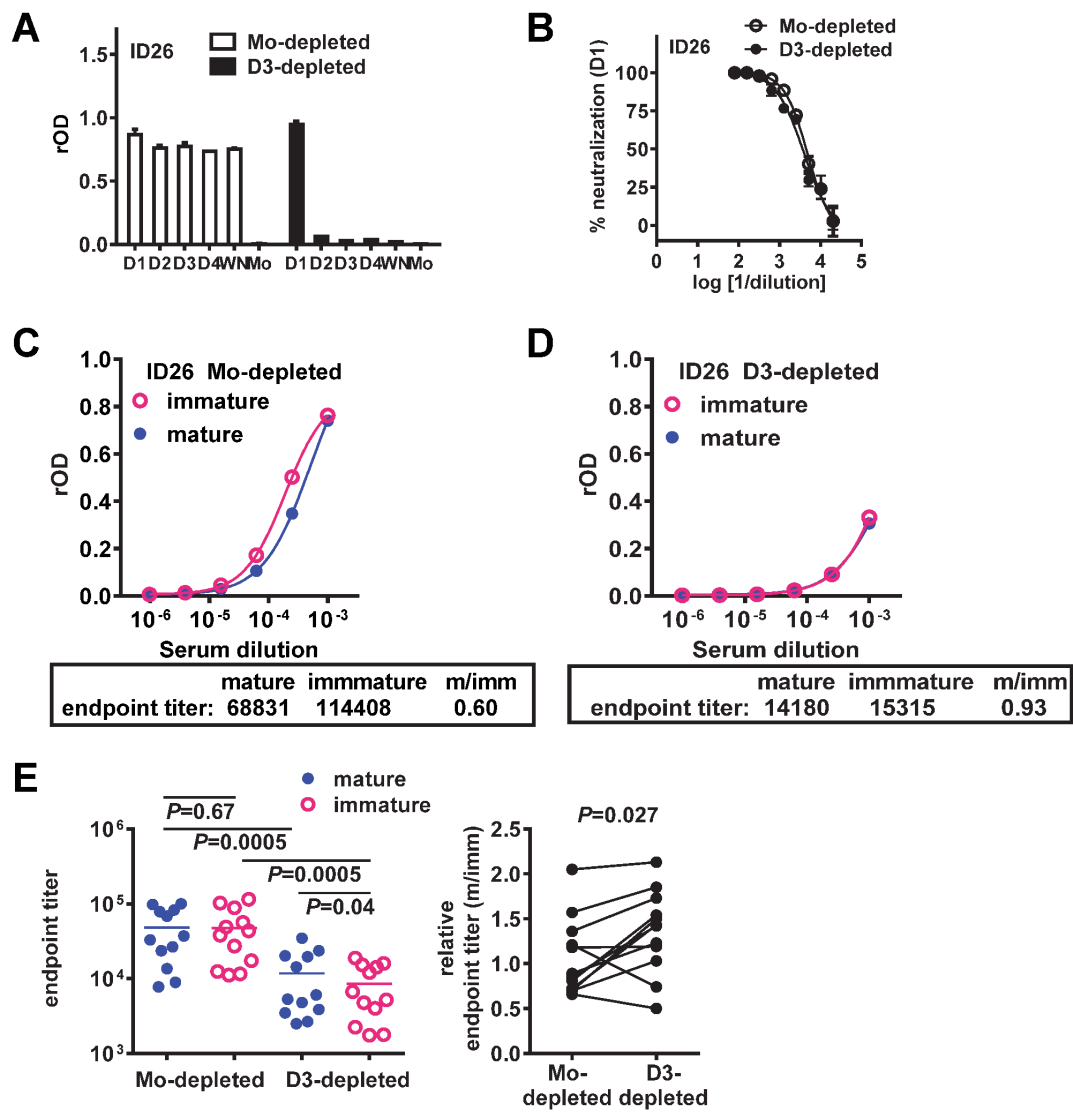


Fig7 revised

