B Cell Responses during Secondary Dengue Virus Infection Are Dominated by Highly Cross-Reactive, Memory-Derived Plasmablasts

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ABSTRACT

Dengue virus (DENV) infection results in the production of both type-specific and cross-neutralizing antibodies. While immunity to the infecting serotype is long-lived, heterotypic immunity wanes a few months after infection. Epidemiological studies link secondary heterotypic infections with more severe symptoms, and cross-reactive, poorly neutralizing antibodies have been implicated in this increased disease severity. To understand the cellular and functional properties of the acute dengue virus B cell response and its role in protection and immunopathology, we characterized the plasmablast response in four secondary DENV type 2 (DENV2) patients. Dengue plasmablasts had high degrees of somatic hypermutation, with a clear preference for replacement mutations. Clonal expansions were also present in each donor, strongly supporting a memory origin for these acutely induced cells. We generated 53 monoclonal antibodies (MAbs) from sorted patient plasmablasts and found that DENV-reactive MAbs were largely envelope specific and cross neutralizing. Many more MAbs neutralized DENV than reacted to envelope protein, emphasizing the significance of virion-dependent B cell epitopes and the limitations of envelope protein-based antibody screening. A majority of DENV-reactive MAbs, irrespective of neutralization potency, enhanced infection by antibody-dependent enhancement (ADE). Interestingly, even though DENV2 was the infecting serotype in all four patients, several MAbs from two patients neutralized DENV1 more potently than DENV2. Further, half of all type-specific neutralizing MAbs were also DENV1 biased in binding. Taken together, these findings are reminiscent of original antigenic sin (OAS), given that the patients had prior dengue virus exposures. These data describe the ongoing B cell response in secondary patients and may further our understanding of the impact of antibodies in dengue virus pathogenesis.

IMPORTANCE

In addition to their role in protection, antibody responses have been hypothesized to contribute to the pathology of dengue. Recent studies characterizing memory B cell (MBC)-derived MAbs have provided valuable insight into the targets and functions of B cell responses generated after DENV exposure. However, in the case of secondary infections, such MBC-based approaches fail to distinguish acutely induced cells from the preexisting MBC pool. Our characterization of plasmablasts and plasmablast-derived MAbs provides a focused analysis of B cell responses activated during ongoing infection. Additionally, our studies provide evidence of OAS in the acute-phase dengue virus immune response, providing a basis for future work examining the impact of OAS phenotype antibodies on protective immunity and disease severity in secondary infections.

Dengue viruses (DENV) cause an estimated 390 million infections worldwide every year (1). With as many as 500,000 cases of severe dengue-related hospitalizations per year, dengue has emerged as one of the most critical arboviral diseases in the world today (2). There are four serotypes of dengue viruses (DENV1 to -4), and each can cause acute infection with a wide spectrum of symptoms (3). Clinical disease can range from self-limiting, mild febrile illness to dengue hemorrhagic fever (DHF) and the fatal dengue shock syndrome (DSS) (3–5). Individuals infected with dengue virus generate serum antibody titers that provide long-term protection against future homotypic infections (6). However, in cases of heterotypic infection, several seroepidemiological studies suggest that prior DENV exposure and preexisting antibody may be risk factors for severe disease (7–11). Furthermore, severe DENV infections typically evolve into DHF/DSS 3 to 7 days after fever onset (3), a time associated with a decline in viremia but a rise in serum antibody levels (12, 13). Consequently, in addition to its role in viral clearance, the humoral immune response has

also been hypothesized to contribute to viral pathogenesis and immunopathology (14, 15).

Several hypotheses have been proposed over the past few decades to explain the increased disease severity associated with DHF and DSS cases. They include excessive T cell responses leading to elevated cytokine levels (cytokine storm), as well as anti-

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TABLE 1 Patient information

Patient	Age (yr)	Gender	Clinical diagnosis ^a	Dengue virus serotype ^b	Fever day of sample ^c	No. of MAbs per patient
31	58	Female	DHF	2	4	14
32	37	Male	DHF	2	3	15
33	14	Male	DHF	2	5	14
39	19	Male	DHF	2	6	10

^a Final clinical diagnosis based on available clinical data according to WHO definitions.

^b Dengue virus serotype determined by RT-PCR.

^c Number of days post-fever onset at which blood was collected.

body-dependent enhancement (ADE) (16–20). The latter implicates preexisting subneutralizing, cross-reactive antibodies in increasing viral uptake, thereby enhancing DENV infection (21, 22). Of the studies that have investigated the involvement of B cells in DENV infection, the majority focus on serum antibody, or memory B cell (MBC), responses in dengue patients a few months to years after viral clearance. Such studies have shown that B cell responses elicited after infection are primarily directed at the structural proteins E and prM and are cross-reactive to multiple serotypes, with a minor proportion exhibiting serotype-specific activity (17, 23–25). While serotype-specific protection is believed to be long term, cross-neutralizing serum titers have been reported to peak a few weeks after infection and to wane within a year (26).

The cellular aspects of the B cell response induced during infection remain less well characterized. We and other groups have shown that a rapid and massive expansion of plasmablasts occurs during the acute phase of human DENV infection (27–29). Plasmablasts can account for as many as 30% of all peripheral lymphocytes in patients a few days to a week post-fever onset (27). This rapidly expanding B cell population, made up almost entirely of DENV-specific IgG-secreting cells, peaks at a time associated with the onset of severe disease symptoms (27). Recently, two groups have investigated plasmablast responses during DENV infection by deriving monoclonal antibodies (MAbs) from patient antibody-secreting cells (ASCs). These studies have shown that the E protein is the primary target of acute-phase B cell responses and that a majority of dengue virus plasmablasts display varying degrees of cross-reactivity. However, questions regarding the origin and the functions of plasmablasts during the dengue virus immune response still remain.

Here, we provide a detailed analysis of the plasmablast response induced during ongoing secondary DENV2 infection in four patients. The acute-phase B cell repertoire contained highly affinity-matured, antigenically selected plasmablasts. Clonal expansions were observed in each of the four donors. We generated a panel of 53 MAbs from sorted patient plasmablasts and found that the majority of DENV-reactive MAbs were E specific. Antibodies that cross-reacted to more than one DENV serotype in both binding and neutralization dominated the MAb panel. A majority of DENV-reactive MAbs, irrespective of neutralization capacity, enhanced DENV infection of U937 cells, indicating that the potential for ADE is not limited to weakly neutralizing antibodies. Interestingly, a large number of MAbs in the panel, including half of all mononeutralizing MAbs, preferentially neutralized DENV1, suggesting that original antigenic sin (OAS) may play a role in the dengue virus immune response. These characterizations of plasmablast-derived MAbs give insight into the specificity and function of early antibody responses in dengue virus infection

at a single-cell level and may further our understanding of the role of antibodies in the pathology of dengue.

MATERIALS AND METHODS

Patient cohort. As part of a larger clinical study of dengue patients admitted to Siriraj Hospital in Bangkok, Thailand, four patients were selected for an in-depth analysis of the dengue virus plasmablast response. The serotype of ongoing infection was determined by serotype-specific reverse transcription (RT)-PCR as previously described (27, 30). Based on dengue virus-specific serum IgG titers and an IgM/IgG ratio lower than 1.7, all four patients were classified as having secondary infections (31). Patient details, including age, sex, date of sampling, and information related to their clinical diagnosis, are outlined in Table 1. These studies were approved by the institutional review boards at Siriraj Hospital and Emory University.

Dengue virus and viral antigens. Dengue virus (DENV1 WP, DENV2 Tonga/74, DENV3 Sleman/78, and DENV4 Dominica/84) were graciously provided by S. Whitehead (NIH). Viral stocks were made by infecting Vero cells (ATCC CRL-1586) in Opti-Pro SF medium (Invitrogen; 12309019). Purified paraformaldehyde (PFA)-inactivated DENV2 particles were purchased from Microbix Biosystems Inc. (DENV2 16681; EL-22-02). DENV1 to -4 recombinant envelope proteins were purchased from CTK Diagnostics (A2301, DENV1 VN/BID-V949/2007; A2302, DENV2 GWL39 IND-01; A2303, DENV3 US/BID-V1090/1998; A2304, DENV4 341750).

Flow cytometry, PBMC isolation, and sorting of plasmablasts. Staining for analytical flow cytometry of plasmablasts was performed as described previously (27, 32, 33). Briefly, 300 μ l of whole blood was stained with the antibodies CD19-fluorescein isothiocyanate (FITC), CD38-phycoerythrin (PE), CD3-peridinin chlorophyll protein (PerCP), CD20-PerCP, and CD27-allophycocyanin (APC), followed by red blood cell (RBC) lysis. The percentage of plasmablasts, defined as CD19⁺ CD3[−] CD20^{−/low} CD27^{high} CD38^{high} cells, among total CD19⁺ B cells was determined. Flow cytometry data were analyzed using FlowJo software. The remaining whole-blood sample was processed to isolate peripheral blood mononuclear cells (PBMCs) for use in enzyme-linked immunosorbent spot (ELISPOT) assays and for plasmablast single-cell sorting (32). The plasmablasts were single-cell sorted into 96-well PCR plates containing RNase inhibitor using a FACSaria III and were frozen immediately on dry ice (32, 33).

ELISPOT assay. The numbers of DENV-specific and total IgG-, IgM-, and IgA-secreting plasmablasts were determined using an ELISPOT assay as previously described (27, 32, 34). In brief, 96-well ELISPOT assay filter plates (Millipore; MAHA N4510) were coated overnight with either purified DENV2 virions (10 μ g/ml) or goat anti-human Ig (Jackson ImmunoResearch; 109-005-064) diluted in phosphate-buffered saline (PBS). PBMCs isolated from whole blood were washed thoroughly, added to the plates, and serially diluted. After overnight incubation at 37°C, biotinylated anti-human IgG (Invitrogen; H10015), anti-human IgA (Invitrogen; H14015), or anti-human IgM (Invitrogen; H15015) was added to the plates for 1.5 h at room temperature, followed by avidin D-horse-radish peroxidase (HRP) conjugate (Vector Laboratories; A-2004) for 1 h. Finally, the plates were developed using 3-amino-9-ethyl-carbazole

(AEC) substrate (Sigma; A5754). The developed ELISPOT plates were scanned and analyzed using an ELISPOT counter (Cellular Technologies Ltd.).

Generation of monoclonal antibodies from plasmablasts. The generation of MAb from single-cell-sorted plasmablasts was performed as described previously (32, 33, 35). Briefly, the Ig gene rearrangements of IgG heavy and kappa and lambda light chains of the sorted plasmablasts were identified and amplified by reverse transcription and PCR. Instead of the one-step RT-PCR described previously, cDNA was synthesized using random hexamers, followed by PCR steps. The first round of PCR was performed using a cocktail of primers that covered all families of variable (V) and joining (J) genes, followed by a nested PCR to determine the sequences of the V and J genes of the heavy and light chains. VDJ/VJ (where D stands for diversity) regions were then amplified by a second PCR step, using highly specific primers containing restriction sites for subsequent cloning. The heavy- and light-chain sequences were cloned into separate plasmid vectors containing a human immunoglobulin constant gene and expressed in HEK293 cells. The MAbs were purified using affinity chromatography with Sepharose A beads (33).

Analysis of clonality and somatic hypermutation of V_H genes. Heavy chain variable region (V_H) sequences generated from single-cell-sorted plasmablasts were analyzed to examine repertoire breadth. The IMGT database (Laboratoire d'ImmunoGenetique Moléculaire [LIGM]) was used to identify the V and J gene families for all the sequences. To determine the frequencies of clonal expansions, all plasmablast-derived V_H sequences (including MAb V_H sequences) were analyzed ($n = 29, 32, 33$, and 24 for patient [Pt.] 31, Pt. 32, Pt. 33, and Pt. 39, respectively). For somatic hypermutation analysis, the V_H mutation frequency refers to the total number of silent and replacement mutations between and including framework region 1 (FR1) and complementarity-determining region 3 (CDR3). The mutation frequencies of naive ($n = 5$), IgG memory ($n = 7$), and IgG germinal center (GC) ($n = 8$) B cells and ASCs during influenza virus infection ($n = 6$) were obtained from previously published data (36).

ELISA. For envelope enzyme-linked immunosorbent assay (ELISA), plates were coated overnight at 4°C with DENV1 to -4 recombinant envelope proteins at 0.25 µg/ml. Virion ELISA was performed with purified DENV2 virions at 1 µg/ml. The plates were washed with PBS with 0.5% Tween (PBS-T) and blocked with PBS containing 10% fetal bovine serum (FBS) and 0.2% Tween (PBS-T-FBS) for 1.5 h. Monoclonal antibodies generated from all four patients were serially diluted in PBS-T-FBS and added to the plates for 1 h. After incubating with a peroxidase-conjugated anti-human IgG antibody (Jackson ImmunoResearch; 109-036-098) for 1.5 h, the plates were developed using *o*-phenylenediamine substrate. For virus capture ELISA, plates were coated overnight at 4°C with mouse anti-flavivirus monoclonal antibody 4G2 (Millipore; MAB10216) diluted 1/1,000 in carbonate binding buffer. After blocking with PBS-T-FBS for 1 h, 50 µl of virus-containing supernatant from infected Vero cells was added to each well. The plates were incubated at room temperature for half an hour and washed 10 times with PBS-T-FBS, and serially diluted MAbs were then added. The addition of the secondary antibody and the developing steps were performed as described above. For all ELISAs, antibody concentrations were plotted versus their respective optical density (OD) values at 490 nm. The minimum effective concentration for binding was determined as the concentration required to obtain three times the signal obtained with plain blocking buffer or an irrelevant MAb.

Neutralization assay. A focus-forming assay (FFA) was used to determine the neutralization potential of MAbs or serum. Serially diluted MAbs/serum were incubated with 100 focus-forming units (FFU) DENV1 to -4 at 37°C for 1 h. The mixture was added onto Vero cell monolayers for 1 h at 37°C. A 2% methylcellulose (Sigma; M0512-2506) overlay was added to the infected cells for 72 h at 37°C. After a 3-day incubation, the cells were fixed with a 1:1 mixture of methanol and acetone and processed for staining. Infected foci were stained using 4G2 for 2 h, followed by HRP-linked anti-mouse IgG (Cell Signaling; 7076S) for 1 h.

Foci were developed using TrueBlue peroxidase substrate (KPL; 50-78-02) and imaged and counted on an ELISPOT reader.

Antibody-dependent enhancement assay. A flow cytometry-based assay was used to determine the infection enhancement capacities of MAbs. First, 5,000 FFU of DENV1 to -4 was mixed with an equal volume of MAb at 1 µg/ml for 1 h at 37°C. The virus and MAb mixture was then added to a 96-well plate containing U937 cells (ATCC; CRL-1593.2) at 20,000 cells/well. After 24 h of incubation at 37°C, the infected cells were fixed with Fix/Perm Solution (BD; 51-2090KZ), permeabilized with Perm/Wash buffer (BD; 51-2091KZ), and stained for 1 h using 4G2 followed by anti-mouse IgG Alexa Fluor 488 (Life Technologies; A11029) for 1 h. The number of infected cells was determined using flow cytometry, and the fold enhancement was calculated as the relative percent infection with virus plus dengue virus MAb treatment compared to the percent infection with virus alone.

RESULTS

Potent plasmablast induction in patients with secondary DENV2 infection. To characterize the dengue virus plasmablast response in depth, four patients with confirmed DENV2 infection were sampled during acute disease. All the patients were clinically diagnosed with DHF based on WHO definitions (37) and were classified as having secondary infections based on serum IgM/IgG ratios (Table 1). Blood samples were collected 3 to 6 days post-fever onset to determine the magnitudes of plasmablast responses. Plasmablasts represented a large percentage of the peripheral B cell population in all four donors, ranging between 51% and 80% of all peripheral B cells (Fig. 1A). The cells primarily produced IgG (data not shown), and a large proportion of the total IgG response was DENV specific in all patients (Fig. 1B and C). These observations related to the timing, magnitude, isotype usage, and specificity of plasmablast responses in dengue patients were all consistent with our previous findings (27). Additionally, serum neutralization assays revealed potent titers toward DENV2, mimicking the ELISPOT data showing high DENV2 reactivity (Fig. 1C and D). Interestingly, serum DENV1 titers in all four patients were also high at this time point.

A majority of patient plasmablast-derived MAbs were DENV reactive. To investigate the roles of plasmablasts and the antibodies they produce during ongoing DENV infection, we generated and analyzed antibodies produced by this effector B cell population. We single-cell sorted plasmablasts into 96-well microtiter plates and PCR amplified their Ig heavy- and light-chain variable regions as we have previously reported (32, 38). These VDJ/VJ sequences were subsequently cloned into IgG expression vectors and expressed as MAbs. We generated a panel of 53 MAbs, with 10 to 15 MAbs from each patient (Table 1). For the initial MAb screen, we performed indirect ELISAs using formalin-inactivated DENV2 whole virions, the same antigen used in the ELISPOT assays. A majority of the MAbs reacted to the DENV2 virions, confirming that the MAbs generated were largely DENV specific (Fig. 1E).

The plasmablast response during secondary DENV infection is highly affinity matured and contains clonal expansions. Although plasmablasts have previously been shown to dominate the B cell response during ongoing dengue virus infection, the origin of these antibody-secreting cells remains unclear. We hypothesized that the plasmablast response during secondary infection is mainly MBC derived, given that a majority of these acute-phase cells are class switched, secrete IgG, and appear in the circulation rapidly after fever onset. To test this, we compared variable gene

FIG 1 Generation of MAbs from plasmablasts of patients with secondary DENV2 infection. (A) Percentages of plasmablasts (defined as CD19⁺ CD3⁻ CD27^{hi} CD38^{hi} lymphocytes) among total CD19⁺ B cells determined by flow cytometry. (B) Representative ELISPOT analysis showing total and dengue virus-specific IgG-secreting cells for Pt. 31 and Pt. 33. Each well shown contained 222 PBMCs. The numbers below the wells are spot counts. (C) Total and dengue virus-specific IgG-secreting cells per million PBMCs for all patients. Above each bar is the percentage of total IgG-secreting cells that were dengue specific. (D) Serum neutralization titers were determined by FFA, and FRNT₅₀ values are shown. FFAs were performed on serum samples collected the same day the plasmablasts were sorted for MAb synthesis. The values are from two or more independent experiments, with the mean FRNT₅₀ plotted. The dotted line represents the maximum dilution factor of serum tested (1:200). Each serum sample was tested against all four DENV serotypes in FFA. (E) The antibodies generated were tested for binding using the same antigen as in the ELISPOT assay. Antibodies from all the patients are shown.

somatic hypermutation (SHM) frequencies in our dengue virus MAb panel with previously published data from other B cell populations (36). We found that plasmablasts from dengue patients had high SHM frequencies, with per-patient V_H mutation averages ranging between 14.5 and 21.7 (Fig. 2A). These levels were comparable to those seen in influenza virus infection (Fig. 2A) and vaccination (32). The number of V_H mutations in dengue patient plasmablasts was significantly higher than that of mutations in GC IgG⁺ B cells and higher, though not statistically significantly, than IgG⁺ memory B cells (Fig. 2A). The levels of SHM were comparable among the four dengue patients, ranging from 5 to 39 mutations per sequence and averaging 18.1 mutations overall (Fig. 2B and Table 2).

Additionally, we examined replacement (R) versus silent (S) V_H mutation frequencies in all the MAb sequences in our panel for signs of selection. A preference for R mutations in the CDRs (R/S ratio, >2.9) suggests antigenic selection. All four patients had an average CDR R/S ratio of >2.9 (Fig. 2C), suggesting that the plasmablasts making the MAbs were antigenically selected as a response to DENV infection. We also analyzed the VDJ/VJ gene usage of the plasmablast heavy- and light-chain sequences for the presence of clonal expansions. An analysis of plasmablast clonality is shown in Fig. 2D. Clonal relatedness between the plasmablast sequences from each patient averaged 23% overall. In three of the four patients, clonal expansions represented more than 20% of all sequences analyzed, reaching as high as 28% in Pt. 32.

Highly cross-reactive, E-specific MAbs dominate the secondary dengue virus plasmablast response. Previous studies have shown that the viral structural protein E is a major antigenic target for human humoral responses to DENV (17, 23, 25, 29). We tested the MAb panel for reactivity to E by performing ELISAs with

DENV1 to -4 recombinant envelope (rE) proteins. We found that a majority of MAbs (31/53) bound to rE of at least one DENV serotype, and all rE-specific MAbs were either partially (2 or 3 serotypes) or fully (4 serotypes) cross-reactive (Fig. 3). No serotype-specific rE binding was observed. Interestingly, we noted striking differences in rE binding patterns between patients. While a majority of Pt. 31 and Pt. 39 MAbs reacted strongly to rE, most MAbs from Pt. 32 and Pt. 33 failed to bind to rE of any serotype (Fig. 3A). The MAbs that did not bind to rE were tested for DENV reactivity by Western blotting using DENV2 lysate. An additional 6 MAbs were found to be E specific (data not shown), making E the target for 70% of the MAbs in the panel.

The MAbs were tested for neutralization against DENV1 to -4 by FFA (Fig. 3 and 4A). A majority of MAbs in the panel (46/53) exhibited neutralizing activity *in vitro*, 34 (74%) of which were either partially or fully cross neutralizing. The cross-neutralizing MAbs from each patient displayed a range of phenotypes, varying in the permutations of serotypes neutralized, as well as in the potency of neutralization of each serotype. Serotype-specific neutralizing MAbs represented 23% of the overall MAb panel. Most of these mononeutralizing MAbs reacted to either DENV1 (5/12) or DENV2 (6/12) (Fig. 3A and 4B). Overall, DENV4 was the least neutralized serotype, as only 20 MAbs showed DENV4-neutralizing activity. As with binding trends, the neutralization patterns of MAbs also differed between patients. Whereas DENV2 was neutralized most potently by a majority of Pt. 31 and Pt. 39 MAbs, DENV1 was the preferred serotype of neutralization for most Pt. 32 and Pt. 33 MAbs (Fig. 3 and 4A).

For most MAbs, neutralization patterns against DENV1 to -4 did not mirror rE binding activity. For instance, 10/12 MAbs from Pt. 31 had comparable affinities for the four DENV rE proteins by

FIG 2 Plasmablasts induced during secondary DENV infection are highly affinity matured and are clonally related. (A) V_H mutation frequency in the dengue patient cohort compared to historical data. Each circle represents the average number of V_H nucleotide mutations per donor. Somatic hypermutation frequencies in naive, memory, germinal center, and influenza virus-specific peripheral B cells were derived from previously published data (36). Statistical analyses were performed using an unpaired, two-tailed t test. *, $P < 0.05$; **, $P < 0.005$. (B) Ranges of V_H mutation frequencies in all four dengue patients. Each square represents the number of V_H mutations per MAb sequence. (C) R/S mutation ratios in the entire V_H gene, CDRs, and FR. The ratios are based on the average numbers of R and S mutations for all MAb sequences per patient. A ratio above 2.9 (dotted line) suggests antigenic selection. (D) Clonality in plasmablast-derived heavy-chain sequences for all the patients. The number at the center of each pie chart is the total number of heavy-chain sequences analyzed (MAb sequences plus unpaired V_H sequences that were not pursued for MAb synthesis). The asterisks indicate clonal groups for which a MAb exists in our panel. The percentage of clonal sequences is also shown.

ELISA but displayed varied neutralization activities against DENV1 to -4 by FFA (Fig. 3A). While 26 MAbs in the panel fully cross-reacted to DENV1 to -4 rE, only 6 of them also neutralized all four DENV serotypes (Fig. 3A). A large number of these fully cross-reactive MAbs were instead shown to neutralize only 2 or 3 serotypes *in vitro*. Additionally, 15 MAbs that did not react to rE by ELISA exhibited neutralizing activity by FFA (Fig. 3A and 4B).

In addition to their neutralizing titers, we also tested the abilities of the MAbs to enhance DENV infection using a human histiocytic lymphoma cell line, U937. These monocytic cells express Fc γ receptors (Fc γ R) and show significantly higher levels of DENV infection in the presence of enhancing antibodies (39). The MAbs were screened for ADE potential against all four DENV serotypes at a concentration of 1 μ g/ml. We found that 45/53 MAbs either moderately (5- to 25-fold) or potently (>25-fold) enhanced DENV infection (Table 3). A majority of MAbs that tested positive for DENV binding and/or neutralization also exhibited ADE activity. Cross-reactive neutralizing MAbs on average caused greater fold enhancement of infection than mononeu-

tralizing MAbs. However, a majority of mononeutralizing MAbs also enhanced DENV infection. In fact, the MAb 31.3F03, which neutralized only DENV2 *in vitro*, exhibited the highest cross-serotypic enhancing activity in the entire MAb panel.

Evidence of OAS in secondary dengue virus immune response. As noted previously, a large number of MAbs in the panel neutralized DENV1 more potently than DENV2 (Fig. 4A). This DENV1-biased phenotype was primarily observed in Pt. 32 and Pt. 33 MAbs. The neutralization bias toward DENV1 for Pt. 32 and Pt. 33 was also reflected at the serum level (Fig. 1D). We also noticed that a majority of DENV1-biased MAbs failed to react to any of the four DENV serotypes by rE ELISA. Conversely, the MAbs that neutralized DENV2 more potently mostly exhibited binding activity to all DENV rE proteins (Fig. 3 and 4B).

These distinct phenotypes in binding and recognition of rE were also observed in serotype-specific MAbs. Except for 31.3G04, which efficiently neutralized DENV4, all the mononeutralizing MAbs clustered as either DENV1 or DENV2 specific for neutralization. Notably, the 5 DENV1-specific MAbs exhibited stronger

TABLE 2 Heavy- and light-chain characteristics of dengue patient-derived monoclonal antibodies

Pt.	MAb	Heavy chain		CDR3 length (aa) ^a	No. of mutations		Light chain			CDR3 length (aa)	No. of mutations	
		V gene	J gene		Total	S/R	Kappa/lambda	V gene	J gene		Total	S/R
31	3C02	1-69	4	20	10	4/6	Lambda	2-23	2	10	7	2/5
	3C04	1-2	4	16	16	3/13	Kappa	3-20	4	9	11	5/6
	3D02	3-23	4	11	23	6/17	Kappa	3-20	3	9	14	5/9
	3D03	4-4	3	21	5	0/5	Kappa	1-5	2	10	7	3/4
	3E03	3-43	4	12	19	5/14	Lambda	7-43	2	9	14	6/8
	3F01	3-30	4	11	23	8/15	Kappa	2-30	4	8	11	2/9
	3F02	3-30	4	13	29	3/26	Kappa	2-30	2	8	2	1/1
	3F03	3-43	4	12	22	7/15	Lambda	7-43	2	9	19	8/11
	3G02	3-13	4	11	15	3/12	Kappa	2-30	4	8	6	2/4
	3G04	3-9	6	20	13	2/11	Lambda	1-40	3	10	9	2/7
	3G05	3-23	4	11	25	7/18	Kappa	3-20	3	9	16	5/11
	3G06	3-30	4	19	18	6/12	Lambda	2-23	2	12	19	4/15
	3H02	3-30-3	4	7	22	6/16	Kappa	3-11	2	9	9	5/4
	3H04	4-30-2	4	17	16	4/12	Kappa	1-12	1	9	13	4/9
32	2C01	4-59	4	12	7	1/6	Kappa	3-15	2	10	5	3/2
	2C02	3-30	4	16	15	5/10	Kappa	1-33	4	10	9	2/7
	2C04	3-48	6	19	13	5/8	Kappa	1D-12	4	10	17	5/12
	2C06	3-72	5	16	8	1/7	Kappa	1-5	1	3	2	1/1
	2D02	4-59	4	14	7	0/7	Lambda	3-21	2	12	16	3/13
	2D03	3-21	3	21	12	5/7	Kappa	1-5	2	5	20	5/15
	2D06	1-46	5	19	12	3/9	Lambda	3-21	2	11	6	1/5
	2E01	3-72	4	14	39	14/25	Kappa	1-6	4	9	15	8/7
	2E02	1-46	6	21	11	1/10	Kappa	2-28	2	9	3	0/3
	2E04	1-46	6	21	17	4/13	Kappa	2-28	2	10	5	1/4
	2E05	1-46	6	20	14	2/5	Kappa	2-28	3	9	2	1/1
	2F02	3-64	4	13	17	1/16	Kappa	3-20	1	9	9	1/8
	2F03	3-48	6	16	23	6/17	Kappa	3-20	4	9	23	4/19
	2F04	1-46	5	12	11	0/11	Kappa	1D-17	4	10	4	1/3
	2G02	1-46	5	19	12	3/9	Lambda	3-21	2	11	6	1/5
33	3A04	1-69	4	19	19	9/10	Kappa	3-11	5	9	14	6/8
	3A06	4-61	5	18	35	13/22	Lambda	1-44	3	11	18	6/12
	3B06	1-46	6	21	25	6/19	Lambda	3-21	3	9	11	2/9
	3C05	1-69	5	20	14	5/9	Lambda	1-40	3	11	11	2/9
	3D02	3-15	4	14	12	1/11	Kappa	3-20	2	9	8	3/5
	3D04	3-49	5	20	22	1/21	Kappa	1-39	1	9	15	0/15
	3E01	1-69	4	14	39	17/22	Kappa	3-15	5	10	13	5/8
	3E04	3-21	5	18	22	6/16	Kappa	3-15	2	10	8	6/2
	3F01	3-21	6	13	25	4/21	Kappa	3-20	3	9	7	1/6
	3F02	5-10-1	3	11	24	7/17	Lambda	1-47	3	11	17	3/14
	3F03	1-69	5	17	25	7/18	Kappa	3-15	5	9	10	4/6
	3F05	3-11	4	10	10	4/6	Kappa	1-33	1	9	10	3/7
	3G01	3-23	4	21	18	5/13	Kappa	3-11	3	10	3	0/3
	3G04	3-21	4	11	14	5/9	Lambda	1-44	3	11	6	1/5
39	3A02	3-7	3	21	31	11/20	Lambda	2-14	2	10	23	8/15
	3A04	4-39	4	11	24	5/19	Lambda	2-14	2	10	12	1/11
	3B02	3-21	4	13	7	1/6	Lambda	1-47	3	11	12	0/12
	3C01	1-69	6	19	24	8/16	Lambda	1-51	2	12	8	3/5
	3D01	3-21	4	13	20	4/16	Lambda	1-47	3	9	13	4/9
	3D02	4-59	5	20	9	3/6	Lambda	1-44	3	11	12	3/9
	3D06	7-4-1	4	9	16	6/10	Lambda	7-43	3	12	32	8/24
	3E06	1-69	5	16	15	5/10	Lambda	2-11	1	13	8	2/6
	3G02	3-30	4	12	24	7/17	Kappa	1-9	4	10	21	7/14
	3G03	3-7	4	16	10	4/6	Kappa	1-39	2	9	8	2/6

^a aa, amino acids.

neutralizing activity against their preferred serotype (median 50% focus reduction neutralization titer [FRNT₅₀], 0.16 μ g/ml) than DENV2-specific MABs (median FRNT₅₀, 1.2 μ g/ml), even though DENV2 was the infecting serotype in all four patients. While the DENV2-specific MABs bound to all 4 rE proteins, all DENV1-specific MABs tested negative for rE binding. We suspected that this lack of binding could be due to the absence of critical conformational epitopes in the rE proteins and therefore attempted a capture ELISA using unfixed, intact virus to probe for binding activity. The capture ELISA confirmed the fully cross-reactive binding phenotype of DENV2-specific MABs. More interestingly, however, it revealed that the MABs that primarily neutralized DENV1 also showed a clear preference for DENV1 in binding. Low levels of binding to DENV2 were also recorded for the DENV1-specific MABs, potentially explaining why the plasmablasts making these MABs were activated in response to the ongoing DENV2 infection.

DISCUSSION

Until recently, B cell responses in dengue patients were largely studied after convalescence, months to years after the resolution of infection. These studies provide valuable insight into the epitopes and varied binding, neutralization, and ADE phenotypes of dengue virus-specific antibodies. However, they fail to capture plasmablasts, the B cell subset responsible for the peak in serum antibody observed during an ongoing dengue virus infection (27). In an effort to understand the origin and functional properties of dengue virus plasmablasts, we isolated plasmablasts from four Thai patients experiencing secondary DHF grade DENV2 infection and generated a panel of MABs by single-cell PCR and cloning of plasmablast Ig genes. Our key findings were as follows. (i) Secondary dengue virus infection induces highly affinity-matured, antigenically selected plasmablasts that exhibit several clonal expansions. (ii) The dengue virus plasmablast response is predominantly E specific and cross-reactive with regard to both binding and neutralization. (iii) Most dengue virus-reactive MABs were capable of enhancing infection *in vitro*, irrespective of the neutralization potency or degree of cross-reactivity. (iv) A large number of MABs demonstrated preferential neutralization of a heterologous serotype, reminiscent of OAS and strongly suggesting an MBC origin for the secondary dengue virus plasmablast response.

All four patients mounted potent DENV-specific plasmablast responses, echoing the findings of our previous study in an independent Thai cohort (27). The plasmablasts primarily secreted IgG only a few days after fever onset. The magnitude and rapidity of antibody production suggested that these plasmablasts may have been induced as a recall response. To gain insight into the origin of the dengue virus plasmablast response in our patient cohort, we analyzed MAB SHM frequencies. We found that dengue virus plasmablasts had a high SHM frequency, comparable to levels seen after annual influenza vaccination (32) or infection

(38). SHM levels in the four dengue patients were higher than those seen after true primary infection or vaccination, such as in acute HIV infection (AHI) patients or primary vaccinia virus vaccinees (38, 40), as well as SHM rates in memory B cells. The high levels of somatic mutation suggest that dengue virus plasmablasts may be reactivated MBCs. A preference for replacement mutations in the CDRs of plasmablast-derived MABs, together with the high frequency of antigen-specific antibodies, indicates that the ASCs were functionally selected. As the plasmablasts used for MAB synthesis were not enriched based on antigen specificity, but rather, collected based on *in vivo* activation, we were able to examine the repertoire of the plasmablast response during the ongoing infection with minimal bias. An analysis of the plasmablast immunoglobulin repertoire revealed clonal expansions in all four donors. Surprisingly, 23% of all dengue patient-derived Ig sequences analyzed were clonal. The levels of clonal relatedness, although lower than those observed after influenza virus vaccination (32), are notably higher than those in naive B cells or MBCs (41). The presence of clonal expansions in all four patients also further supports an MBC origin of the plasmablast responses.

In the past few years, several groups have generated panels of human MABs from MBCs of dengue virus-infected patients and have shown that multisero-type-reactive MABs dominate the dengue virus immune response (17, 23–25, 42). The surface glycoprotein E has been described as an important target in the human immune response to DENV infection (23, 25, 42, 43). More recently, two groups derived MABs from dengue patient ASCs and demonstrated that the E protein contains major epitopes for antibody binding and viral neutralization during ongoing infection (29, 44). As expected, a majority of MABs (31/53) in our panel bound to rE. All rE-reactive MABs displayed cross-reactivity to two or more DENV serotypes, and a large number of MABs also neutralized more than one DENV serotype *in vitro*. Given that all the patients were previously exposed to DENV and that the four DENV serotypes share high genomic sequence homology (5), we were not surprised by the large number of fully cross-reactive MABs in our panel. A recently published report examining secondary responses in dengue vaccinees ably demonstrates the importance of cross-reactive antibodies and the impact of their depletion on serum neutralization and binding titers (45). Our data showing the large abundance of cross-reactive MABs during natural dengue virus infection reiterates the significance of broadly neutralizing responses to vaccine design efforts.

For a majority of MABs, the patterns of binding and neutralization did not fully overlap. Of the 26 MABs that fully cross-reacted to all four DENV rE proteins, 6 MABs were serotype specific for neutralization. The remaining 20 neutralized 2 or 3 serotypes by FFA. These data suggest that the recognition of conserved epitopes by antibodies is not sufficient for effective neutralization of multiple DENV serotypes. Conversely, several MABs that were DENV specific by capture virus ELISA and/or neutral-

FIG 3 Patient plasmablast-derived MABs display highly heterogeneous binding and neutralization activities. (A) Each MAB was tested against all four dengue virus serotypes for binding and neutralization activity. To test binding, ELISAs were performed with rE proteins from DENV1 to -4. The values plotted represent the minimum concentrations required to obtain three times the background signal obtained with plain blocking buffer. Neutralization activity was determined by FFA, and FRNT₅₀ values are shown. FFAs were performed with duplicates in two or more independent experiments, and the mean value is plotted. The dotted lines represent the maximum concentrations of MABs tested in ELISA (5 μ g/ml) and FFA (20 μ g/ml). (B) Summary of rE binding patterns of MABs as determined by ELISA. Each bar represents one patient, and the sections within the bar indicate the extents of cross-reactivity, as shown in the legend on the far right. (C) Summary of DENV neutralization patterns of MABs as determined by FFA. FRNT₅₀ values of ≤ 5 μ g/ml were considered a positive result for neutralization. The bars are coded as for panel B.

FIG 4 Secondary DENV2 infection induced the activation of DENV1-specific plasmablasts in patients. (A) MAb FRNT₅₀ values against DENV1 to -4 as determined by FFA. Each circle represents one MAb, and the solid lines connect the same MAb across serotypes. The dotted lines represent the highest concentration of MAbs tested in the FFA (20 μg/ml). Antibodies below the dotted line failed to neutralize the virus by 50% at 20 μg/ml. (B) Comparison of binding versus neutralization activities of mononeutralizing MAbs. Binding was tested by rE ELISA and capture virus ELISA. 31.3H04, shown on the left of the dashed line, is provided as a contrasting example of a MAb with full cross-reactivity. Each MAb was tested against all four DENV serotypes in ELISA and FFA.

TABLE 3 ADE activities of monoclonal antibodies

ADE Fold Increase at 1 µg/mL						ADE Fold Increase at 1 µg/mL					
Fully cross-reactive (4 serotypes) binding to rE	Mab	DENV1	DENV2	DENV3	DENV4	Partially cross-reactive	Mab	DENV1	DENV2	DENV3	DENV4
	31.3D02	-	5	5	5		33.3A06	3	23	17	4
	31.3F01	28	35	25	36		39.3G02	-	4	3	-
	31.3F03	21	40	65	40		32.2C06	-	2	-	2
	39.3B02	7	15	14	11	Non-reactive by rE ELISA	31.3G04	16	3	-	4
	39.3D06	14	34	19	46		32.2C02	2	7	-	2
	39.3G03	-	-	-	-		33.3C05	2	16	4	-
	31.3D03	-	4	5	2		33.3E04	18	5	-	-
	31.3E03	11	28	12	26		33.3F03	2	27	6	-
	31.3F02	23	53	24	36		39.3E06	8	3	-	6
	31.3G02	9	31	16	161		32.2D06	14	42	39	3
	31.3G05	-	3	5	2		32.2E04	20	48	50	6
	31.3G06	10	18	9	22		32.2E05	11	57	48	45
	31.3H02	10	20	13	24		33.3A04	14	2	2	14
	32.2D03	14	4	5	6		33.3B06	24	66	11	-
	32.2E02	15	67	39	9		33.3E01	18	12	15	-
	32.2G02	3	84	57	-		33.3F02	24	8	-	-
	33.3D02	21	20	32	20		33.3G01	3	10	8	-
	33.3D04	3	38	28	-		39.3D02	23	33	5	-
	33.3F01	3	10	39	6		32.2D02	8	19	31	30
	33.3F05	13	17	41	17		31.3C02	4	-	-	13
	33.3G04	12	21	35	22		32.2C01	-	-	-	-
	39.3A04	9	27	13	23		32.2C04	-	-	-	-
	39.3C01	3	4	3	6		32.2E01	-	-	-	-
39.3D01	-	2	4	9	32.2F02		28	21	-	-	
31.3H04	24	35	54	49	32.2F03		-	-	-	-	
39.3A02	19	35	19	30							
32.2F04	-	-	-	-							

Serotype-specific in neutralizationPartially-cross neutralizingFully cross-neutralizingNon-reactive

Serotype-specific in neutralization
 Partially-cross neutralizing
 Fully cross-neutralizing
 Non-reactive

ized at least one DENV serotype failed to react to rE. This could be explained by potential antigenic differences between the laboratory-adapted strains used in our *in vitro* assays and the clinical strains the patients were infected with at the time of the study. Another possibility is the absence of critical conformational epitopes in rE that are found only in the context of the whole, intact virion. Recent studies have shown that human MAbs with potent DENV neutralization ability can recognize conformational epitopes positioned on the E dimer interface (44, 46). These MAbs do not bind to monomeric rE proteins, as the epitopes they recognize are unique to the E dimer. It is possible that the MAbs in our panel that do not bind rE exhibit similar conformation sensitivity or bind to epitopes expressed only on an intact virion.

An alternative hypothesis is that some of the MAbs in the panel are actually prM specific or, in the case of nonneutralizing MAbs, NS1 specific. Previous studies have shown the abundance of prM- and NS1-reactive memory B cells in convalescent dengue patients (17, 23–25). Western blots using DENV2 lysate revealed that none of the MAbs had reactivity to these proteins (data not shown). The absence of binding to NS1 and prM in our assays could be due to antigen-based limitations, and therefore, the possibility that some of these MAbs bind non-E epitopes cannot be completely ruled out at this time. Future binding and epitope-mapping experiments testing the rE-nonreactive MAbs will be useful in determining where these antibodies bind.

While all four patients were diagnosed with DENV2 infection at the time of the study, the neutralization patterns of the MAbs generated varied greatly between patients. A large proportion of Pt. 32 and Pt. 33 MAbs neutralized DENV1 more potently than DENV2, echoing the DENV1 bias also observed at the serum level for these two patients. Given that all the patients were experiencing secondary infection, we suspected that the neutralization bias

toward DENV1 in the two patients could be due to a previous DENV1 exposure. Screening the DENV1 mononeutralizing MAbs by captured virus ELISA revealed that these MAbs had a clear preference for DENV1 in binding, in addition to neutralization. The low levels of binding to DENV2 detected validated our hypothesis that these plasmablasts resulted from the reactivation of MBCs from a past DENV1 exposure, rather than as part of a naive response to the current DENV2 infection. OAS has been well described for antibody responses to repeated influenza virus exposures (47–50), and a few reports have demonstrated this phenomenon in the secondary dengue virus B cell response, as well (29, 51, 52). Our data strongly suggest that OAS may play a role in the secondary dengue virus immune response and result in decreased binding affinity and neutralization potency against the infecting serotype. However, the physiological relevance of OAS and its impact on protective B cell responses in dengue needs to be examined further, both in animal models and in humans.

In the case of patients with a history of DENV exposure, epidemiological data suggest that progression to the more severe forms of disease is often associated with secondary heterotypic infection (22, 53, 54). ADE is one of several hypotheses proposed to explain this observed increase in dengue disease severity (53, 55). To address whether plasmablast-derived antibodies were capable of infection enhancement, we determined the fold increase in DENV infection of FcγR-bearing cells in the presence of the MAbs in our panel. With the exception of 32.2F04 and 39.3G03, every DENV-reactive MAb demonstrated enhancing ability *in vitro*. Regardless of the degree of cross-reactivity or the potency of neutralization, a majority of MAbs increased DENV infection at 1 µg/ml. Although these MAbs had the capacity to enhance DENV infection, the likelihood that plasmablast responses contribute to ADE during the current infection is low. This is because viremia in

dengue patients starts to subside before the peak in B cell responses (12, 13, 53, 56). The generation of plasmablast responses with ADE potential is likely more critical for future dengue virus exposures, as enhancing antibodies may already be present in serum at the time of viral infection.

In conclusion, we have shown that the B cell response rapidly generated after secondary DENV infections contains plasmablasts with memory B cell origin. The plasmablasts induced are highly affinity matured and produce antibodies that are largely cross-reactive, E specific, and capable of potent DENV neutralization *in vitro*. However, in addition to the cross-reactive MABs, we report the presence of several type-specific neutralizing MABs, including a subset that more potently neutralized a heterotypic strain than the infecting serotype. Various degrees of cross-reactivity and neutralization were observed across the panel, showcasing the vast heterogeneity in the plasmablast responses of secondary dengue patients. Future epitope-mapping experiments with the most potentially neutralizing MABs may reveal important targets for prophylactic and therapeutic interventions. Moreover, focused analyses of the DENV1 versus DENV2 mononeutralizing MABs may provide valuable information about the different epitopes recognized by these MABs and how they impact DENV serotype specificity and neutralization. Additionally, a less explored yet interesting avenue is the comparison of primary and secondary plasmablast responses. A study comparing MBC-derived MABs from primary and secondary infected patients suggested notable differences in cross-reactivity, avidity, neutralization potency, and mechanisms of neutralization between primary and secondary infections (42). Future clinical studies involving comparisons of secondary versus primary cases or patients from areas of nonendemicity may provide added insight into the biology of plasmablast responses and their role during the acute dengue virus immune response.

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