Rapid and massive virus specific plasmablast responses during acute dengue virus infection in humans

Running title: Plasmablast responses to dengue infection

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Humoral immune responses are thought to play a major role in dengue-induced immuno-pathology, however little is known about the plasmablasts producing these antibodies during an ongoing infection. Herein we present an analysis of plasmablast responses in patients with acute dengue virus infection. We found very potent plasmablast responses that often increased more than 1000 fold over the baseline levels in healthy volunteers. In many patients these responses made up as much 30% of the peripheral lymphocyte population. These responses were largely dengue virus-specific, almost entirely made up of IgG secreting cells and reached very high numbers at a time after fever onset that generally coincided with the window where the most serious dengue-induced pathology is observed. The presence of these large, rapid and virus-specific plasmablast responses raises the question as to whether these cells might have a role in dengue immuno-pathology during the ongoing infection. However, it also illustrates the need for a more detailed understanding of the repertoire and specificity of the antibodies that these cells produce.
INTRODUCTION

Dengue virus causes an infection with symptoms ranging from a mild fever to severe hemorrhagic fever, with vascular leakage that ranges in severity from minor subcutaneous bleeding to severe gastrointestinal bleeding (5, 28, 29). A striking epidemiologic and immunological characteristic of dengue fever is that the severe immunopathology is more likely to occur in individuals who have previously been infected with a heterologous dengue serotype (8, 30, 33). While the exact mechanism of this phenomenon remains to be fully elucidated, several hypotheses have been developed over the last few decades to explain the reason for the exacerbated pathology observed in these patients. One of the main hypotheses revolves around a mechanism referred to as antibody-dependent enhancement (ADE) (14). This hypothesis suggests that during a secondary infection, cross reactive yet poorly cross-neutralizing antibodies produced against a previously encountered serotype will mediate an increased infectivity in addition to altering the host range of target cells. This mechanism has been extensively studied in vitro (6, 17, 20) and its importance in vivo is beginning to be elucidated (2, 10, 27). Another proposed hypothesis (22, 23) suggests that an enhanced infection together with a potent T cell mediated recall response produce massive amounts of effector mediators (4, 11-13, 15, 16, 25), a so-called cytokine storm, that is responsible for the observed immunopathology. These two mechanisms are not mutually exclusive and may in fact work in concert to cause the immunopathology of dengue disease.
While human T cell responses during acute dengue infection have been studied in some detail, much less is known about the B cell responses. Early studies in dengue patients showed that increases in immunoglobulin containing cells could be observed during infection and that these cells reached maximal numbers near the subsidence of fever (7). It has also been shown that total CD19+ B cells increase during dengue infection and that these increases correlate with the presence of so-called atypical lymphocytes (19). In addition, a more recent study of the global gene-expression patterns in peripheral blood PBMCs isolated from DHF patients showed an enrichment of plasmablast signatures that was accompanied by an increase of plasmablasts by flow cytometric analysis (31).

Herein, we have analyzed the magnitude, kinetics, antigen specificity and isotype usage of the plasmablast responses induced in pediatric and adult patients with acute dengue infection. We found a very potent and rapid induction of virus-specific plasmablasts during the acute phase of the infection, which in some cases made up as much as 30% of total lymphocytes. The rapid expansion of plasmablasts was observed in the infected patients at a timepoint that generally coincides with the subsidence of fever and the most serious symptoms. These findings suggest that these cells, and the antibodies that they produce, might be involved in dengue immunopathology. However, while suggestive, these findings also clearly illustrate the need for more detailed analyses of the plasmablasts and the antibodies they produce during the acute phase of dengue infection to clearly define their potential role in dengue immunopathology.
MATERIALS AND METHODS

Dengue patient cohort

Patients enrolled in this study were diagnosed clinically with dengue virus infection upon admission to Siriraj Hospital in Bangkok, Thailand. The dengue infection was confirmed by a serotype specific RT-PCR as well as several other diagnostic tests (NS1 test, dengue-specific IgG and IgM test (ELISA or dip-stick tests)). Routine laboratory measurements (CBC, urine and blood chemistry) and clinical manifestations of dengue infection were recorded. A final diagnosis and severity classification were done at the conclusion of the trial with a full review of all the clinical and laboratory data. Information about the patient cohort is detailed in Table 1 and in Supplemental Table 1. All studies were pre-approved by the Faculty of Medicine at Siriraj Hospital and the Emory institutional review boards.

PBMC and plasma isolation

Peripheral blood mononuclear cells (PBMC) and plasma were essentially isolated as previously described (35). Briefly, blood samples were collected in Vacutainer CPT tubes (Becton Dickinson, BD). Plasma samples were isolated from the CPT tubes and preserved at -80 °C. The PBMCs were collected and washed extensively, and resuspended in PBS with 2% FCS for immediate use or frozen in liquid nitrogen in FCS (fetal calf serum) with 10% DMSO for subsequent analysis.
Serological determination of primary or secondary dengue infection

Dengue IgM and IgG antibodies were detected by a modified ELISA as described by Innis et al. (18). Briefly, microtiter plates (NUNC) were coated with 100 μl of goat antihuman IgM or IgG and stored at 4°C. Serum specimens were diluted at 1:100 (0.05% PBS-T, 3% skim milk), added and incubated for 1 hour at 37°C. After washing, 50 μl of pooled dengue antigens diluted at 1:3 (0.05% PBS-T, 2% lipid extracted NHS, 3% skim milk) was put into each tested well, incubated for 1 h at 37°C and washed before adding 50 μl of anti-dengue complex (2H2) monoclonal antibody diluted at 1:10000. After 1 h at 37°C, plates were washed before adding 50 μl of the goat anti-mouse conjugate. Plates were read at 492 nm using an ELISA reader (BioTek Powerwave 340) and samples were considered positive at the 0.500 OD absorbance.

Identification of dengue virus serotype

Dengue virus RNA was extracted from patient plasma using QIAamp viral RNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer’s instruction. Dengue virus serotype was determined by a multiplex nested polymerase chain reaction (PCR) method as previously described (36) with some modification. Briefly, RNA was subjected to reverse transcription (RT) with primer DEUR (5'-GCTGTGTCACCCAGAATGGCCAT-3') and a multiplex nested PCR with four primer pairs specific for the E region of each dengue serotype was performed. Four pairs of serotype specific primers were used including D1L (5'-GGGGCTTCAACATCCCAAGAG-3') and D1R (5'-GCTTAGTTTCAAGTGCTTTTT
CAC-3’) for DENV-1, D2L (5’-ATCCAGATGTCATCAGGAAAC-3’) and D2R (5’-CCGGCTCTACTCCTATGATG -3’) for DENV-2, D3L (5’-CAATGTGCTTGAATACCTTTGT-3’) and D3R (5’-GGACAGGCTCTCCTTTCTTG-3’) for DENV-3 and D4L (5’-GGACAACAGTGGTGAAGCA-3’) and D4R (5’-GGTTACACTGTTGATTTCTCA-3’) for DENV-4. The expected sizes of the nested PCR products for DENV-1, DENV-2, DENV-3, and DENV-4 were 504 bp, 346 bp, 196 bp, and 143 bp, respectively.

**Viral antigen**

Purified DENV-2 particles were purchased from Microbix Biosystems Inc. (Canada, Cat. no: EL-22-02). According to the manufacturer, this preparation contains virus particles (Den-2, strain 16681) concentrated from tissue culture supernatants by precipitation and ultracentrifugation. The antigen is then purified by sucrose density gradient centrifugation. Virus particles are separated from the sucrose-containing buffer by ultracentrifugation and the antigen is resuspended in Medium 199 followed by inactivation at room temperature with formaldehyde. For the final product formulation the formaldehyde is neutralized by the addition of sodium bisulphite.

**Analytical flow cytometry**

Staining for analytical flow cytometry of plasmablasts was performed on whole blood as previously described (35). Briefly, cells were stained with the appropriately titrated antibodies, followed by lysis of erythrocytes (BD FACS
Lysing Solution, Pharmingen, Cat. no: 349202) and fixing in 2% PFA. All antibodies were purchased from Pharmingen (CD19-FITC (555412), CD38-PE (555460), CD3PerCP (340663), CD20PerCP (347674)), except anti-CD27 APC (17-0279-73) which was purchased from eBiosciences. Plasmablasts were defined herein as CD19^+CD3^-CD20^-lowCD27^highCD38^high cells, gated on an extended lymphocyte gate to include blasting cells. Flow cytometry data was analyzed using FlowJo software. Absolute cell numbers per volume blood were calculated using the BD True count Tubes bead system (BD Biosciences, Cat. no: 340334) according to the manufacturer's protocol.

**ELISPOT assay**

Direct ELISPOT to enumerate the number of either dengue specific or total IgG, IgM and IgA secreting plasmablasts present in the PBMC samples were performed as previously described (9, 35). Briefly, 96-well ELISPOT filter plates (Millipore, MAHA N4510) were coated overnight with either the optimized amounts of purified dengue virions as described above (10ug/ml) or with polyvalent goat anti-human immunoglobulins (10ug/ml) (Jackson Immuno Research, Cat. no: 109-005-064) in PBS. Plates were washed and blocked by incubation with RPMI containing 10% FCS at 37°C for 2 hrs. Purified and extensively washed PBMCs were added to the plates in dilution series starting at 10 x10^5, diluted 3-5 fold down the plate and incubated overnight. Plates were washed with PBS followed by PBS containing 0.05% Tween and then incubated with either a biotinylated anti-human IgG antibody (Invitrogen, Cat. no: H10015),
anti-human IgA (Invitrogen, Cat. no:H14015) or anti-human IgM (Invitrogen, Cat. no: H15015) for 1.5 hrs at room temperature. After washing, the plates were incubated with an avidin-D-HRP conjugate (Vector Laboratories, Cat. no: A-2004) and finally developed using AEC substrate (3 amino-9 ethyl-carbazole, Sigma). Developed plates were scanned and analyzed using an automated ELISPOT counter (CTL, Cellular Technologies Ltd.).

**ELISA**

Direct ELISA was performed by coating ELISA plates overnight with either anti-human Ig (Jackson Immuno Research, Cat. no: 109-005-064) or dengue antigen (as described above) at 10 mg/ml. Plates were washed with PBS with 0.5 % Tween and blocked in PBS with 10% FCS and 0.2% Tween. Serially diluted serum samples were then added to the plates, followed by incubation with a peroxidase conjugated anti-human IgG antibody (Invitrogen, Cat. no: H10015) and finally developed using OPD substrate. Serum dilution was plotted versus OD value at 492 nm and the midpoint dilution values were determined.

**Statistical analysis**

Data was collected and graphed using MS Excel and Graphpad Prism software. Data for each individual subject is presented as circle dot with a bar that represents the median of each group. An unpaired, two-tailed t test was used to determine the statistical significance of the difference observed between groups.
Spearman correlation coefficient test was used to analyze the correlation observed between different parameters.
The patient cohort in this study was comprised of individuals diagnosed with dengue virus infection at Siriraj Hospital, Bangkok, Thailand in 2009 to 2011. Single blood samples were obtained 2 to 8 days after symptom onset and for some of the donors also at a convalescent timepoint 1 month or later after discharge. Of 56 patients diagnosed clinically, 46 were confirmed as infected with dengue virus (Table 1 and Supplementary Table 1), while 10 were found to have some other, non-dengue, febrile illness. Those testing positive for dengue were infected with dengue 1, 2 or 3, while none were infected with the dengue 4 strain. This result is in concordance with previous studies demonstrating the low prevalence of DENV-4 in the area (21). Of the 46 confirmed dengue cases, 28 were classified as having DF (dengue fever), 17 DHF (dengue haemorrhagic fever) class I and II and one patient had DSS (DHF class III) according to the WHO criteria (28). Three additional patients were also enrolled in 2011, where we obtained two consecutive blood samples during hospitalization. Serological analyses showed that of the 46 dengue patients only 4 were mounting primary responses, based on a IgM/IgG ratio lower then 1.7 (18) (Supplemental Table 1). Thus, the majority of the patients enrolled in the study had had previous exposure to dengue virus and were mounting secondary immune responses.

Potent and rapid plasmablast responses induced by dengue infection

Antibodies are thought to play a central role in the development of dengue immunopathology (2, 6, 10, 20). However, relatively little is known about the
magnitude or kinetics of the B cell responses induced during the acute phase of
dengue infection. Fig 1A shows representative examples of the flow cytometric
analysis of plasmablasts (35) during the acute infection. These patients
demonstrated a massive induction of peripheral plasmablasts, in some cases
corresponding to as much 30% of peripheral lymphocytes. Analysis of the overall
set of patients showed that the majority had very high numbers of plasmablasts
(Fig 1B), averaging 47% of the peripheral B cells. At convalescence, one month
after discharge, the numbers of peripheral plasmablasts had returned to baseline
levels, similar to that found in healthy adults.
The magnitude of this response is striking particularly when compared with the
immune responses to influenza booster vaccination (peaks day 7, historical data
from (35)) or primary infection (peaks day 11-14) with the live-attenuated yellow
fever virus vaccine. While these results are derived from clinical studies
performed in the US (1, 24, 35) and therefore in different cohorts and settings, it
is clear that the already sizeable plasmablast responses observed in both these
systems ((35) and Fig 1D) are significantly smaller in magnitude compared to the
responses observed during dengue virus infection.
In terms of absolute numbers, several dengue patients carried more than 1x10^6
plasmablasts per ml of blood (Fig 1 C and 2A) (median: 3.7x10^5), which is a 1000
fold increase over what is normally observed in healthy individuals. Not
surprisingly, these responses are of a similar magnitude regardless of what
serotype of dengue virus the patients were infected with (Figure 1C). Although
this trial was not designed as a detailed kinetic analysis, the timing of the
samples we analyzed provided interesting information about the kinetics of this response. Samples obtained on day 2-3 generally displayed relatively low numbers of plasmablasts, often indistinguishable from samples taken from healthy adults. However, as shown in Fig 2A, the samples obtained from patients later after symptom onset showed a steady and dramatic increase of plasmablasts, reaching peak numbers by day 6-7. This finding is substantiated by the analysis of a small number of additional donors in a separate trial where we found similar increases in consecutive blood samples taken from the same donor. Thus Figure 2B shows an analysis of three donors, with consecutive blood samples obtained 2 days apart, where we saw a significant increase of plasmablasts when comparing the earlier timepoint to the later one. The clinical diagnosis of dengue fever (DF) or dengue haemorrhagic fever (DHF) did not correlate with the magnitude or kinetics of the plasmablast response (Fig. 2A) although this might have been confounded by the fact that all patients were unwell enough to require hospital admission. Finally, the responses observed in the four donors who mounted primary responses (based on serological analyses) were similar to those observed in the secondary responders (Supplementary Figure 1).

We conclude from these analyses that dengue infection induces a massive plasmablast response, often leading to increases of more than 1000 fold over the number observed in healthy donors. This increase occurs rapidly over several days after fever onset with very high numbers reached around day 6-7, in some
cases generating a plasmablast pool that makes up as much as a third of all peripheral blood lymphocytes.

The majority of the plasmablast response during the acute phase of dengue virus infection is dengue specific

It has been proposed that infection can lead to activation not only of antigen specific cells but also that the pro-inflammatory environment could lead to the activation of both B and T cells in a non-specific manner, i.e. bystander activation (3, 32, 34). To determine the extent to which the dengue infection-induced plasmablast response was the result of antigen-specific or non-specific bystander activation we examined the specificity and isotype usage of the antibodies they produced using a dengue specific ELISPOT (Fig 3A). This analysis showed that the response was dominated by dengue specific IgG secreting cells, with several donors showing more than $10^5$ dengue-specific IgG secreting cells per $10^6$ PBMCs. We also observed dengue specific IgA secreting cells in most donors but at about a 100 fold lower frequency. Finally, a small number of the donors had detectable IgM secreting plasmablast responses. Importantly, none of these were IgM only, and only one (donor Den01-066) had an IgM response that was larger than the IgG response in the same donor. It is interesting to note that of the four donors identified as primary responders, all had detectable IgM responses by ELISPOT. Of these, donor 066 mentioned above had a very high serum IgM/IgG ratio (Supplemental Table 1) and also showed the highest IgM response by ELISPOT.
Comparing the number of specific and total IgG secreting plasmablasts we found that the majority of the IgG response was indeed dengue specific, often with 70% or more of the IgG secreting cells being specific for the virus (Fig 3B and C). This frequency is likely an underestimation, as these assay were performed using formalin inactivated purified Den-2 virus. A large proportion of these responses are likely directed against proteins or epitopes shared between the dengue serotypes as no significant difference was seen in the frequency of virus specific cells between patients infected with dengue 2 versus those with Den 1 or Den 3 infections (Supplemental figure 2). The current study was aimed at understanding the overall immune responses induced by dengue infection, but future studies will be aimed at understanding the sero-type specific responses and their functional properties. Fig 3B also shows two patients with acute Hepatitis A infection or an unidentified febrile illness, respectively. Both patients showed substantial plasmablast responses, however, none of the induced cells were dengue specific. When samples were analyzed from patients returning one month after hospital discharge, plasmablast numbers had returned to baseline. This illustrates that these responses are transient in peripheral blood, likely reflecting substantial cell death as well as migration to tissues capable of long-term maintenance of plasmacells, such as the bone marrow.

Analyzing the serological responses induced at the same timepoint as the plasmablast analyses showed that there were high titers of dengue specific IgG present during the acute phase of the infection (Figure 4A). The infected patients all had very high levels of dengue specific antibody, but there was a wide
variability between individuals. Of interest, the two patients with primary infection (based on the IgG/IgM ratios) showed very low overall titers at this timepoint. Furthermore, as expected, among the healthy adult controls from Thailand 2/3 had detectable titers whereas the healthy controls from the US were seronegative. Finally, comparing the dengue specific serological responses to the plasmablast responses we found that they correlated well as shown in Figure 4B and C.

Given the size and isotype of the plasmablast response it might be expected that these patients show evidence of hypergammaglobulinemia. To this end we also measured the total IgG in these patients and healthy controls. However, no significant differences were observed (data not shown) between the patients and the healthy controls.

Collectively, this study shows that dengue infection induces a very potent virus-specific plasmablast response in nearly all of the patients (93%). These responses are dominated by IgG secreting cells and increased as much as 1000 fold in magnitude reaching maximal numbers by day 6-7 after fever onset. These findings regarding the magnitude and specificity of the plasmablast responses during the acute phase of dengue infection prompts more in depth analyses of plasmablast responses with regards to repertoire breadth and fine specificity of the antibodies they produce, as well as their possible involvement in dengue-induced immunopathology.
DISCUSSION

In an effort to analyze the plasmablast responses induced during the acute phase of dengue virus infection, we have examined a cohort of patients identified at Siriraj Hospital in Bangkok, from both pediatric and adult clinics. This patient group contained both patients with mild (DF) and more severe (DHF) symptoms. We found very potent plasmablast responses induced in almost all of the dengue infected donors. In many cases these responses completely dominated the B cell compartment (often as much as 80% of the CD19+ B cells were plasmablasts), making up as much as 30% of the total peripheral lymphocytes. In contrast, by way of comparison to US volunteers receiving the influenza vaccine (35) or a primary vaccination with the yellow fever vaccine we find much smaller responses, on the order of 2-3% of the total CD19+ B cells. The magnitude of the plasmablast response during dengue infection correlated with the time following the onset of febrile symptoms. Thus, most samples taken 2-3 days after fever onset showed barely detectable response, but increased over time to the massive responses observed in samples obtained on days 6-7. These findings were substantiated by additional analysis of three donors from whom two samples were obtained, 2 days apart, which showed a similar increase with time. Importantly, ELISPOT analysis showed that the majority, often 80 percent or more, of the dengue-induced plasmablasts were secreting dengue virus-specific IgG. This suggests that these cells were induced by interaction with their cognate antigen, and were not the result of non-specific, bystander mechanisms. Interestingly, even though we used a DENV-2 virus clone for detection of antigen
specific cells in the ELISPOT, there was little difference in relative frequency of antigen specific cells over total IgG secreting cells in patients infected with DENV-1 or 3 as compared to patients infected with DENV-2 (Supplementary Figure 2). This indicates that most of the plasmablast cells induced by infection are specific for proteins or epitopes present in multiple serotypes. Future studies, on a monoclonal level, are needed to identify what protein/epitope these responses target, what proportion of these responses is functionally active and how this correlates with serotype specificity.

Patients returning to the hospital for a follow-up visit around 1 month after discharge showed essentially baseline numbers of plasmablasts by flow cytometry with no dengue virus specific cells by ELISPOT. This demonstrates that these cells are only present in peripheral circulation for a relatively short time, likely representing either contraction through extensive cell death or migration of at least a subset of these cells to tissues where long term antibody production can be sustained, such as bone marrow or secondary lymphoid tissues. Furthermore, while patients suffering from other febrile illnesses often showed sizeable numbers of plasmablasts in the periphery, these cells were not dengue specific by the ELISPOT assay.

The patient cohort studied herein almost exclusively represented secondary exposures to dengue, based on serological analyses (Supplemental Table 1) as well as the fact that the virus is endemic in this area. In fact, the vast majority of the population in this region sero-converts very early in life (8). Although the few primary cases identified in the current cohort showed similar responses to the
overall cohort, they were too few to reach a solid conclusion regarding differences between the responses induced in primary versus secondary responses. Ongoing studies of primary dengue infection in non-endemic areas will determine how these data compare to primary immune responses in terms of magnitude, kinetics and isotype distribution in the plasmablast response.

Acute infection with dengue virus results in symptoms ranging from a mild fever to severe vascular leakage and massive internal bleeding. Interestingly, epidemiological studies suggest that the immunological history might be important for the outcome of infection, such that while a primary exposure to this virus generally leads to a mild disease and life long protection against that serotype, a subsequent exposure to a heterologous strain can sometimes lead to the more severe form of dengue, with the accompanying haemoragic symptoms described above. Both T and B cell mediated mechanisms have been implicated in dengue-induced immunopathogenesis, but the importance of either of these mechanisms have not been fully characterized in vivo (23). Recently, two papers have also described dengue specific memory B cells at convalescent timepoints and characterized the antibodies that the memory B cells produce (2, 10). However, studies describing the antibodies produced by responding plasmablasts during the acute phase of the infection have not been reported. Ongoing studies to clearly define the repertoire breadth of the infection-induced plasmablasts, the epitopes they recognize, and their ability to cause immunopathology by ADE-like responses (antibody dependent enhancement) at a monoclonal level will provide further insight into the role of these cells.
In addition to their role in binding antigen, antibodies are able to regulate immune responses through interaction with several different Fc-receptors, a function that is directly dependent on their Fc region glycosylation pattern. Recent studies (reviewed in (26)) have clearly shown that regulated glycosylation patterns on IgG can mediate pro- or anti-inflammatory functions. These findings suggest a mechanism for ensuring that steady-state serum IgG maintains an anti-inflammatory state while, in contrast, upon antigenic challenge by a pathogen the antigen specific IgG antibodies produced can mediate a more pro-inflammatory state. Given the large ASC responses that we have observed in dengue patients it is possible that the IgG produced by the plasmablasts are also contributing to pathology by exacerbating the pro-inflammatory state in these patients. However, while the dengue infected patients showed very high titers of dengue specific antibody that were proportional to the magnitude of the plasmablast response (Figure 4), no overall increases of total IgG were found in these patients (data not shown). Given the massive numbers of plasmablasts present in these patients during the acute phase of infection this would indicate that only a smaller number of them are able to survive long term as long-lived plasmacells. It is unclear if this is due to that the bone marrow can only sustain a smaller number of plasmacells or if the majority of the induced plasmablasts are pre-destined to a short life span.

In conclusion, human dengue infection induces a very rapid and potent plasmablast response, often dominating the B cell compartment of these patients. We show that these responses are largely dengue specific, almost
entirely made up of IgG secreting cells, and that they reach very large numbers at a time after fever onset that generally coincides with the window where the most serious dengue-induced pathology is observed. The findings are suggestive of a role for these cells in dengue immunopathogenesis. However, more detailed analyses of the fine specificity and functional properties of the antibodies that they produce would be needed to truly address this important question.
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REFERENCES


FIGURE LEGENDS

Figure 1. Potent plasmablast responses induced during acute dengue virus infection. Plasmablast responses in peripheral blood were measured by flow cytometry in healthy controls or in patients diagnosed with dengue fever (retrospectively confirmed as dengue). A subset of the patients came back for a follow-up blood sample one month after discharge. A) Representative flow cytometric analysis of the plasmablast frequency in four patients during ongoing dengue virus infection and one month after resolution. Plasmablasts are defined herein as CD19+/CD3-/CD20-/low/CD38hi/CD27hi cells. All plots shown are gated on CD3-/CD20-/low lymphocytes, including blasting cells. The numbers in the plots represent the percent of that gate, while numbers in parenthesis are percent of all lymphocytes. B) Summary of the frequency of plasmablasts, as a fraction of total CD19+ B cells, in samples obtained from healthy adults, patients with ongoing dengue virus infection and dengue patients returning after at least one month after clearance of infection. Statistical analysis was performed using an unpaired, two-tailed t test. C) Magnitude of the plasmablast response as absolute numbers in patients infected with different dengue viral serotypes. Statistical analysis was done using an unpaired, two-tailed t test. D) Comparison of the magnitude of the dengue virus infection induced plasmablast responses to those observed after either the inactivated influenza virus vaccine (recall response that peaks at 7 days after vaccination (35)) or the live attenuated yellow fever virus (YFV) vaccine (primary response that peaks at day 11/14 after vaccination). Statistical analysis was done using an unpaired, two-tailed t test.
Figure 2. Rapid expansion of plasmablast responses during acute dengue virus infection. A) Absolute plasmablast numbers per ml blood (calculated using a bead based (TruCount; BD) system) for all the donors analyzed as a function of days after fever onset. Patients classified as DHF are shown in red while DF patients are shown in black. Spearman correlation coefficient test was used to analyze the correlation observed between different parameters for all the datapoints. Similar results were obtained when each sub-group was analyzed individually. B) Plasmablast responses in peripheral blood were measured by flow cytometry in three patients diagnosed and confirmed with dengue fever infection on two consecutive timepoints with 2 days between samples. Plots show cells gated for CD3-/CD20-/low cells and is gated as CD27hi/CD38hi cells. Frequency of that gate is shown and frequency of total lymphocytes is shown in parenthesis.

Figure 3. The majority of the plasmablasts induced by dengue virus infection are virus-specific. Plasmablast responses in peripheral blood were measured by ELISPOT analysis during infection and one month after discharge. A) Graph shows the number of IgG, IgA or IgM secreting dengue-specific plasmablasts per million PBMC. ELISPOT plates were coated with purified 16681 Den-2 virion antigen. B) Representative IgG specific ELISPOT analysis of two dengue virus-infected patients, two patients with other acute febrile illness, a dengue patient returning for a 1-month follow-up and a healthy control. Upper row shows dengue specific IgG spots and the lower row shows total IgG.
secreting cells. From left to right the numbers of cells plated was: 823, 823, 2489, 7407, 66000 and 66000 PBMCs. Numbers given below each well is the spot-count for that well. Relative frequency (%) of dengue specific IgG secreting plasmablasts over total IgG secreting plasmablasts is also shown. C. Summary of the number of total IgG secreting cells and dengue specific IgG secreting cells for all the donors analyzed. The entire bar (black and white together) represent the total number of IgG secreting cells per million PBMC while the dengue specific IgG secreting cells are marked in black only. Shown above each bar is the percentage of dengue virus-specific cells over total IgG secreting cells.

**Figure 4. Dengue specific serum antibody correlates with the observed plasmablast responses.** ELISA analysis of serum samples obtained from the acutely infected dengue patients as well as healthy control samples. This groups of samples contained 17 cases of secondary infection, 2 primary cases (based on IgG/IgM ratios as per above), three healthy thai and two healthy US volunteers. Dengue specific IgG was measured by direct ELISA using the same dengue antigen used for the ELISPOT analysis of the cellular responses above. A) ELISA measurement of total dengue specific IgG. Midpoint dilution values were determined as the intersection of the dotted line at 50% OD value, and the ELISA binding curves. B and C) Correlation of the serological responses (as determined by the midpoint dilution value determined in A) and the plasmablast response by absolute plasmablasts in blood (flow cytometry) and dengue specific plasmablasts by ELISPOT.
Table 1. Summary of dengue patients enrolled in the study. a

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<td>Total</td>
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<tr>
<td>Male/Female</td>
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Dengue virus serotype e

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a. Patients were diagnosed based on serology (dengue-specific RT-PCR, NS1 test, dengue specific IgG/IgM test, clinical measurements (CBC, urine and blood chemistry)) and clinical manifestations of dengue infection.
b. One of the 18 DHF cases were classified as DSS (DHF III).
c. Median age is shown with the range in parenthesis.
d. Median day after fever onset on which the blood sample was obtained.
e. Serotype determination by RT-PCR. Four patients were negative by this assay but classified as dengue based on other clinical measurements as described above.
Figure 1.

A. Dengue patients

Return visit (+1 mo)

#1

#2

#3

B. Healthy adults

Dengue patients

Return visit

0

20

40

60

80

100

% plasmablasts of total CD19+ B cells

p<0.0001

p<0.0001

C. Plasmablasts per ml blood

ns ns

D. % plasmablasts of total CD19+ B cells

Healthy adults

Influenza

YFV

Dengue

Den1 Den2 Den3 ND

10

1

10

2

10

3

10

4

10

5

10

6

10

7

ns
A. Plasmablasts per ml blood

Day of fever
0 2 4 6 8

10^3
10^4
10^5
10^6
DHF
DF
p<0.0001

Donor Den02-031

Donor Den02-032

B.

Figure 2.
Figure 4

A

Dengue specific IgG (log 10 of midpoint dilution) vs Serum dilution

B

Plasmablasts per ml blood (log 10) vs Dengue specific IgG (log 10 of midpoint dilution)

C

Dengue specific IgG (log 10 of midpoint dilution) vs Dengue specific plasmablasts per 10^6 PBMC (log 10)