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Antibody epitopes identified in critical regions of dengue virus nonstructural 1 protein in mouse vaccination and natural human infections

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Abstract

Dengue is a global public health problem and is caused by four dengue virus serotypes (DENV1-4). A major challenge in dengue vaccine development is that cross-reactive anti-DENV antibodies can be protective or potentially enhance disease via antibody-dependent enhancement (ADE). DENV nonstructural protein 1 (NS1) has long been considered a vaccine candidate as it avoids ADE. Here, we evaluated survival to challenge in a lethal DENV vascular leak model in mice immunized with NS1 combined with alum, Monophosphoryl Lipid A + AddaVax (MA), or Sigma Adjuvant System + CpG DNA, compared to mice infected with a non-lethal dose of DENV2 and mice immunized with ovalbumin (negative control). We characterized antibody responses to DENV-1, -2 and -3 NS1 using an antigen microarray tiled with 20-mer peptides overlapping by 15 amino acids and identified 5 regions of DENV NS1 with significant levels of antibody reactivity in the NS1+MA group. Additionally, we profiled the antibody responses to NS1 of humans naturally infected with DENV2 or DENV3 in serum samples from Nicaragua collected at acute, convalescent and 12-month timepoints. One region in the “wing” domain of NS1 was immunodominant in both mouse vaccination and human infection studies, and two regions were identified only in NS1-immunized mice; thus, vaccination can generate antibodies to regions that are not targeted in natural infection and could provide additional protection against lethal DENV infection. Overall, we identified a small number of immunodominant regions, which were in functionally important locations on the DENV NS1 protein and are potential correlates of protection.

Keywords

Antibody profiling; dengue virus; NS1; peptide; epitope; immunodominant

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INTRODUCTION

The four dengue virus serotypes (DENV1-4) are transmitted by *Aedes aegypti* and *Ae. albopictus* mosquitoes, causing up to an estimated 390 million infections and 96 million cases of dengue annually (1). DENV is an enveloped flavivirus whose positive-sense 10.7-kb RNA genome encodes a polyprotein that is co- and post-translationally cleaved by host and viral proteases into 3 structural (C, capsid; prM/M, membrane; E, envelope) and 7 non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) proteins. DENV infections in humans range from asymptomatic to dengue fever (DF) to dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) (2). DF is an acute febrile illness with headache, retro-orbital pain, myalgia, arthralgia, rash, hemorrhagic manifestations, and/or leukopenia. The hallmark features of DHF consist of thrombocytopenia, hemorrhagic manifestations, and signs of plasma leakage, which can lead to hypotensive shock (DSS) and death (3). A primary infection with one DENV serotype is thought to lead to homologous protective immunity; however, subsequent infection with a different serotype (secondary heterologous DENV infection) is a major risk factor for severe disease. The antibody response to DENV in both primary and secondary infection is dominated by anti-E antibodies (4, 5), with some anti-prM/M and anti-NS1 antibodies (6–11). Antibody-dependent enhancement (ADE) is thought to occur when cross-reactive anti-E and anti-prM antibodies facilitate entry of DENV into Fcγ receptor-bearing cells, leading to increased viral load, immune activation and more severe disease. In contrast, antibodies to NS1 should not enhance infection as they do not target structural proteins on the virion.

NS1 is the only viral protein secreted by DENV-infected cells, and it is conserved approximately 79% across the four DENV serotypes (12–14). NS1 has been reported to play an immunomodulatory role via its interaction with the complement component C4b (15, 16). While natural infection with DENV has been shown to elicit a potent NS1 antibody response in both humans and mice, some of these antibodies have been shown to cross-react with platelets or endothelial cells (17–19). Although some groups have suggested that these cross-reactive antibodies to NS1 contribute to pathogenesis (8), mice vaccinated with recombinant DENV NS1 are protected against lethal infection without evidence of pathogenesis from antibodies to NS1 (20–22). Overall, NS1 vaccines have been shown to be highly immunogenic in mice, and antibodies to NS1 prevent the pathogenic effects of secreted NS1 (22). In addition, several immunodominant B-cell NS1 epitopes in naturally infected mice are conserved across all four DENV serotypes (23).

A principal challenge in vaccine development is eliciting long-lived memory $CD8^+$ T cells and protective antibodies (24, 25). One effective strategy is the use of adjuvants that enhance vaccine immunogenicity. For example, Monophosphoryl Lipid A (MPLA) adjuvant is a TLR4 agonist that has been shown to elicit strong antibody and $CD8^+$ T cell immune responses and has been approved for human use in the HPV vaccine (26).

Antigen microarrays are high-throughput antibody binding assays that build upon the technology of DNA microarrays. Antigen microarrays have previously been used in several application domains, including identifying immunogenic responses to pathogens with large

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genomes (27, 28) and biomarkers for cancers (29, 30) and autoimmune diseases (31, 32). While antigens spotted on the arrays can include peptides, proteins or virions, we and others have recently shown that peptide antigen microarrays (hereafter referred to as peptide microarrays) spotted with overlapping peptides of influenza glycoproteins can be used to profile vaccine-induced responses in mice and that the antibody profiles generated by distinct vaccines differ from one another (33–35).

Here, we used a peptide microarray spotted with overlapping 20-mer peptides that span the NS1 proteins of three DENV serotypes (DENV1-3) to obtain antibody profiles of mice vaccinated with recombinant DENV2 NS1 combined with three different adjuvants. For comparison, we also mapped the antibody responses to NS1 in the sera of mice infected with DENV2. We then used the same peptide microarrays to profile the NS1 antibody response in humans naturally infected with DENV. We identified five immunodominant regions of antibody reactivity, three of which were shared across DENV serotypes and two of which were recognized by immunized mice, infected mice and infected humans.

MATERIALS AND METHODS

Ethics statement

Animal experiments were performed strictly following guidelines of the American Veterinary Medical Association and the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The University of California, Berkeley's Animal Care and Use Committee pre-approved all experiments. The protocols for the studies involving human subjects were reviewed and approved by the Institutional Review Boards of the University of California, Berkeley, and the Nicaraguan Ministry of Health. Parents or legal guardians of all participants provided written informed consent, and participants 6 years of age and older provided assent.

Viruses

All viruses were propagated in the *Ae. albopictus* C6/36 cell line (American Type Culture Collection; ATCC), titered by plaque assay on baby hamster kidney cells (BHK21, clone 15) and expressed as plaque-forming units (pfu) per ml. The parental strain DENV2 PL046 was obtained originally from H.-Y. Lei (National Cheng Kung University, Taiwan), and DENV2 D220 was produced from PL046 as previously described (36, 37).

Recombinant NS1 proteins

Recombinant NS1 protein from DENV2 was obtained from Merck. The NS1 working stocks were tested using the Endpoint Chromogenic Limulus Amebocyte Lysate (LAL) QCL-1000TM kit (Lonza) and confirmed to be bacterial endotoxin-free (<0.1 EU/ml per 25 ug of protein).

Mouse NS1 vaccination experiments

Six-to-8-week old interferon α/β receptor-deficient 129 mice (A129) were bred and maintained in specific pathogen-free conditions at the University of California, Berkeley Animal Facility. A129 mice were injected intraperitoneally (i.p.) 3 times (days 0, 14 and 42)

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with 20 ug NS1 or 20 ug of ovalbumin (OVA) protein in combination with 1 μg MPLA (InvivoGen, San Diego, CA) and AddaVax (0.5% sorbitan trioleate, 5% squalene, 0.5% Tween-80 in 10 mM sodium citrate buffer; Invivogen) or Sigma Adjuvant System (Sigma Aldrich) with 20 μg CpG or 120 ug aluminum hydroxide and magnesium hydroxide (Imject Alum, Pierce). Two weeks after the third immunization (day 56), mice were challenged intravenously (i.v.) with a lethal dose of 10^7 PFU of DENV2 strain D220. A separate group of mice were infected with 10^5 PFU DENV2 strain PL046 at day 0 as a primary infection control and challenged at day 56. After challenge, mice were observed every 12 hours (h) using a morbidity scoring system on a scale of 1 to 5 (36). Mice were euthanized immediately when they became moribund (score of 5). Immune serum was collected by submandibular bleed from NS1- or OVA-immunized mice or DENV2 PL046-infected mice on day 49.

NS1 ELISA

To determine levels of NS1-specific antibodies in immunized mice prior to challenge, 50 μl of 0.5 μg/ml NS1 was coated onto a Nunc Immulon polystyrene plate and after blocking with 5% nonfat dry milk in PBS+0.05% Tween-20 (PBS-T), 50 μl of a 1:100 dilution of each test serum was added and incubated for 24 h. After washing with PBS-T, biotinylated goat anti-mouse IgG (Jackson ImmunoResearch) was added, followed by washing and addition of streptavidin-alkaline phosphatase (Life Technologies), and para-nitrophenyl phosphate (Sigma-Aldrich) substrate. The reaction was terminated with 0.5 M EDTA after 10–15 minutes, and the optical density was read at OD_{405}.

Human samples

A prospective study was conducted from 2005 to the present in the Infectious Disease Ward of the Hospital Infantil Manuel de Jesús Rivera in Managua, Nicaragua, to study clinical, immunological and viral risk factors for severe dengue. Infants and children between six months and 14 years of age with fever or history of fever <7 days and one or more of the following signs and symptoms: headache, arthralgias, myalgias, retro-orbital pain, positive tourniquet test, petechiae, or signs of bleeding were eligible to participate in the study. Both inpatients and outpatients were enrolled each year during the peak of the dengue season (August 1 to January 31) and followed clinically through the acute phase of illness. A blood sample was collected daily for three days for complete blood counts with platelets, blood chemistry, and serological, virological and molecular biological tests for DENV infection. A convalescent blood sample (14–28 days post-onset of illness) was also collected for paired serological testing. After undergoing a separate informed consent, subjects could choose to participate in a longitudinal arm of the study, wherein blood samples were collected 3, 6, 12, and 18 months post-onset of illness. A participant was considered positive for DENV infection when laboratory tests met one or more of the following criteria: 1) dengue viral RNA was detected by RT-PCR (38, 39); 2) dengue virus (DENV) was isolated (38); 3) seroconversion of DENV-specific IgM was detected by MAC-ELISA in paired acute and convalescent samples (38, 40); and 4) DENV-specific antibody titer by Inhibition ELISA (41–43) demonstrated a 4-fold or greater increase between acute and convalescent sera. Primary DENV infections were considered those in which the convalescent antibody titer was <2,560, and secondary infections were considered those in which the convalescent

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antibody titer was 2,560 as determined by Inhibition ELISA. Laboratory-confirmed dengue cases were classified by severity using a computerized algorithm that compiled all clinical data meeting the criteria for Dengue Fever (DF), Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS), defined according to the 1997 WHO classification criteria (44, 45). Specifically, we tested patient samples collected in 2006–2010 that consisted of 10 DF cases (4 DENV2 primary, 3 DENV3 primary and 4 DENV3 secondary infections), 7 DHF cases (2 DENV3 primary and 5 DENV3 secondary), and 3 DSS cases (DENV2 secondary).

The Nicaraguan Pediatric Dengue Cohort Study is an ongoing prospective cohort study of ~3,500 active participants two to fourteen years of age in Managua, Nicaragua, that has been ongoing continuously since 2004 (46). Every year, a blood sample is collected from all participants to test for and identify any DENV infections that have occurred in the preceding year, while all suspected dengue cases and undifferentiated fevers are worked up to diagnose symptomatic DENV infections. A child whose paired annual serum samples demonstrate seroconversion (a titer of <1:10 to 1:10) or a 4-fold rise in antibody titer as determined by Inhibition ELISA are determined to have experienced a DENV infection. If a child entered the cohort DENV-naïve and experienced no DENV infections during the study, they were considered to be DENV-negative, and their serum samples were used as negative controls in this study.

Peptide synthesis and microarray printing

Twenty-amino acid (aa) peptides with 15-aa overlap between adjacent peptides spanning the DENV NS1 proteins were generated from consensus sequences computed from an alignment of 42 DENV1 (2004–2009), 181 DENV2 (1999–2009–2008) and 123 DENV3 (2008–2010) sequences from viruses collected in Nicaragua. The peptide arrays were designed to map human responses from Nicaraguan patients, and as there is very little circulation of DENV4 in Nicaragua, we generated peptides only to DENV1, DENV2 and DENV3. Two hundred and four peptides (68 for each DENV serotype) were synthesized with a MultiPep RS (IntavisAG) peptide synthesizer using a modified SPOT synthesis protocol. The peptides were lyophilized in a 384-well microtiter plate and stored at −20°C. Peptides were resuspended in 12.5μl DMSO and 12.5μl of ultra-pure water to create a working solution of approximately 1 mg/ml. Peptide stocks were diluted 1:4 in protein printing buffer (3X saline sodium citrate, 0.1% polyvinyl alcohol, and 0.05% Tween-20). Peptide integrity was confirmed using 2 positive controls, Hemagglutinin A (HA) (YPYDVPDYA) and FLAG (DYKDDDDK), included in multiple replicate wells for the initial optimization of the array printing conditions. Peptide stocks were diluted 1:2 in protein printing buffer (PBS with 0.005% Triton X-100) and printed in triplicate on N-hydroxysuccinimide ester-derivatized glass slides (H slides; Schott/Nexterion AG) using a Q-array II microarrayer (Genetix) with contact microarray pins (SMP2.5B; TeleChem). During printing, relative humidity was maintained at 50–60%. Following printing, slides were left to dry overnight. Arrays were stored at −20°C.

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Microarray hybridization

Printed grids were outlined with a PAP hydrophobic pen (Research Products International). Slides were chemically blocked using 4 ml of 50 mM borate, 50 mM ethanolamine for 1 h. Slides were then washed twice with PBS containing 0.05% Tween 20, twice with PBS, and once in deionized water and then spun to dry at 1000xg for 5 minutes at room temperature. Mouse serum samples were diluted 1:25 in 1% BSA and 0.025% Tween 20, incubated on slides for 2 h in a humidified chamber at 25°C, then washed twice with PBS containing 0.05% Tween-20 and twice with PBS. Bound IgG was detected for 45 minutes with Alexa Fluor 647 goat anti-mouse IgG (115-605-008; Jackson ImmunoResearch). Human plasma samples were diluted 1:100 in 1% BSA and 0.025% Tween-20, incubated on slides for 2 h in a humidified chamber at 25°C, then washed twice with PBS containing 0.05% Tween 20 and twice with PBS. Bound antibodies were detected for 45 minutes with Alexa Fluor 647 goat anti-human IgG (Jackson ImmunoResearch). Arrays were washed and spun dry as described above.

Slide scanning

Slides were scanned on a two-laser GenePix 4400A scanner (Molecular Devices) to detect IgG responses. Images were analyzed by GenePix version 7.2 to obtain the mean fluorescence intensity (MFI) for each probe. Responses below 500 MFI after subtraction of background were considered negative (MFI range, 0–65000). Subsequently, all data were analyzed with Matlab (Mathworks) and Python. For background normalization, samples of naïve mice and DENV-naïve humans (annual samples from the Nicaraguan Pediatric Cohort Study, see above) were used as negative controls to adjust for background. For each probe, we computed the median naïve response and subtracted it from the median responses in NS1-vaccinated mice or from the dengue-positive human samples, accordingly.

Unsupervised data clustering

To analyze clustering of antibody profiles in the mice, the responses to the 68 NS1 peptides of DENV2 Nicaragua consensus sequence were used to define a response vector for each animal. Response vectors were compared using the Spearman rank-order correlation measure to create a similarity matrix in which each entry measures the similarity between the pair of response vectors from a specific pair of animals. The samples were then clustered using complete-linkage, a hierarchical unsupervised technique for clustering data points using a given similarity measure (47). Since there were four experimental groups of mice, the number of clusters was arbitrarily set to 6, allowing the existence of outlier clusters. The same methodology was also used to cluster the human samples.

Identifying antigens with differential responses between groups

To compare response rates for specific NS1 antigens from the three DENV serotypes, we first filtered the set of antigens in a treatment-blinded manner, removing all antigens with an overall response rate of less than 20%. The remaining antigens were then individually tested for differences using Fisher's exact test. Non-responders were any response below 5,000 MFI after subtraction of background. To adjust for multiplicity, we computed the family-wise error rates using Bonferroni. Antigens with adjusted p-values < 0.05 were reported. We

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used this methodology to compare the responses of mice in the NS1+Alum vs. NS1+MA groups and also to compare responses of acute, convalescent and 12-month samples of human subjects.

Summary statistics of peptide array data

The antibody profile generated by the peptide microarrays is a multi-dimensional measurement of the antibody responses to a large set of overlapping peptides from NS1. To compare the antibody responses of each sample as measured by the peptide microarray to NS1 in different treatment groups of mice and in different human clinical severity groups across time, we defined the "magnitude" and "breadth" of responses to each protein as follows.

We denote the normalized array measurements by x_{i,s,a_s} where:

i denotes subject, $i = 1, \cdots, N$; s denotes consensus sequence for each DENV serotype, $S = \{DENV1, DENV2, DENV3\}$; a_s denotes peptide antigens from the consensus sequence of each serotype s, $a_s = 1, \cdots, N_s$; z_i denotes treatment assignment (Alum, MA, SCpG, DENV2, etc.) of subject i; y_i denotes outcome of subject i (vaccine-induced antibody titer/infection status/disease status). The observed data for each subject are (z_i, y_i, x_{i,s,a_s}), for $i = 1, \cdots, N$, $s = \{DENV1, DENV2, DENV3\}$ and $a_s = 1, \cdots, N_s$

1. $m_{i,s}=\sum_{a_s=1}^{N_S} x_{i,s,a_s}$ denotes the magnitude of responses to all peptide antigens from the consensus sequence of each serotype s.

2. $b_{i,s}=\sum_{a_s=1}^{N_S} I(x_{i,s,a_s}>0)$ denotes the breadth of response to peptide antigens from the consensus sequence of each serotype s (where positivity is determined using responses of negative controls).

Statistical analysis

To assess baseline levels of responses to the NS1 peptide arrays, we profiled the responses of samples from naïve mice and DENV-negative human subjects. We then used these profiles to normalize the responses of all samples by subtracting the median naïve response for each antigen. This was done separately for mice and human samples. Statistical comparisons between the response magnitudes of the different groups were performed using a Wilcoxon rank sum test. In the mouse challenge experiment, comparison of survival rates was conducted using a non-parametric log rank (Mantel-Cox) test and graphed as Kaplan-Meier survival curves.

RESULTS

Specific adjuvants combined with NS1 provide protection against lethal DENV infection

In order to evaluate the effect of various adjuvants on the ability of NS1 to provide protection, we vaccinated mice three times with 20 μg DENV2 NS1 combined with MPLA +AddaVax (MA), Sigma adjuvant system+CpG DNA (SCpG), aluminum and magnesium hydroxide (alum), or 20 μg OVA combined with MA and then challenged the mice with a

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lethal dose of DENV2 (D220 strain) and measured survival (Fig. 1A). Mice immunized with NS1 in combination with MA or SCpG were protected from lethal challenge, whereas mice immunized with NS1+alum or OVA+MA were not protected. Mice infected with $1x10^5$ PFU of DENV2 (PL046 strain) were protected against lethal challenge because a primary infection with DENV2 serves as a homotypic immunization and protects against a secondary infection even with a lethal challenge dose. Therefore, the vaccine-induced protection was specific to NS1 plus MA or SCpG adjuvant combinations.

Sera collected from the immunized mice and infected mice were tested for NS1-specific IgG by ELISA (Fig. 1B). As expected, the highest levels of NS1-specific IgG were found in mice immunized with NS1 in combination with MA or CpG; much lower levels were detected in mice immunized with NS1+alum, and mice infected with a sublethal dose of DENV2 mice generated intermediate levels of IgG to NS1.

Distinct antibody profiles were generated by NS1 vaccination with different adjuvants or DENV infection

To further characterize the antibody responses in mice immunized with recombinant NS1 combined with different adjuvants or with a low-dose DENV2 infection, we used an antigen microarray spotted with overlapping peptides covering NS1 from the consensus sequences of DENV1, DENV2 and DENV3 Nicaraguan isolates. Each NS1 protein was represented using 20-mer peptides, overlapping by 15 amino acids. Antibody profiles were generated by applying to the arrays individual serum samples collected from mice in three experimental groups: NS1+alum, NS1+MA and NS1+SCpG, as described above. We also profiled responses of mice immunized with OVA as a negative control, as well as mice infected with a low dose of DENV2. We found that mice immunized with the NS1+alum generated weak but detectable antibody responses to the DENV2 NS1 protein, while the groups immunized with NS1+MA or NS1+SCpG generated robust responses to DENV2 NS1 (Fig. 1C), in accordance with the NS1 antibody titers presented in Fig. 1B. Specifically, we computed the overall magnitude of antibody responses in the serum of each mouse, combined the responses in all the mice (see Methods), and compared the 3 different vaccine groups. We found that mice immunized with NS1+MA had significantly higher responses as compared to mice in the NS1+alum group ($p=0.00005$, Fig. 1C). The sera from DENV2-infected mice demonstrated lower reactivity to NS1 peptides than NS1+MA or NS1+SCpG-vaccinated mice.

To further illustrate the differences in antibody profiles of the different treatment groups, we used an unsupervised clustering algorithm to group the antibody profiles of the mice to the DENV1,2,3 NS1 peptide antigens. The algorithm uses the vaccine-induced antibody profiles of each animal in the study to identify mice with similar antibody profiles, and does not make use of the treatment assignment of each animal (see Methods for details). We found that animals in different treatment groups clustered in distinct clusters (Fig. 1D and Supplementary Fig. 1). Overall, the clusters of mice immunized with NS1+MA and NS1+SCpG were closely related in a separate branch from DENV2 infection; in turn, this entire branch was separated from the alum cluster.

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NS1 vaccine-induced antibody responses target specific NS1 epitopes

Since we observed that the overall magnitude of responses in the NS1+MA group was significantly higher than the NS1+alum group, we next turned to identify the specific peptide antigens that were targeted more robustly in the NS1+MA group. Comparisons were performed using Fisher's exact test, and values were adjusted for multiplicity for each strain separately, using Bonferonni. The set of antigens tested for each strain was blindly filtered to include only regions in which the response rates were > 20% (see Methods). Since there were only two animals in the SCpG group, we did not perform statistical comparisons with this group. Using the DENV1, DENV2 and DENV3 peptide arrays, we identified 5 DENV NS1 regions in which responses of the NS1+MA group were significantly stronger than those of the NS1+alum vaccinated mice (Table I, Fig. 2A–C). The five regions of NS1 antibody reactivity are: Region 1 (aa 21–40), Region 2 (aa 101–135), Region 3 (aa 156–175), Region 4 (aa 231–255), and Region 5 (aa 296–335).

DENV2 NS1 vaccination generated cross-reactive responses to all three DENV serotypes and increased the breadth of the antibody response compared to DENV2 infection

While mice were vaccinated with DENV2 NS1, we profiled their responses to the NS1 protein of DENV1-3 in order to detect serotype cross-reactive responses. We compared NS1+alum-and NS1+MA-induced responses to each of the three DENV serotypes separately. Several of the regions were independently identified in more than one serotype. Most notably, antibody responses to Region 2 were cross-reactive to all three serotypes tested and also to DENV2-infected mice (Supplementary Fig. 2). The antibody responses to Region 1 were also cross-reactive across all three DENV serotypes, but were statistically identified in DENV1 and DENV3 only. The responses to Regions 3 and 5 were immunodominant in DENV2 serotype only, with only weak reactivity to these regions in DENV1, and responses to Region 4 were only observed in DENV3. To assess whether these cross-reactive responses are due to sequence conservation in these regions, we generated sequence alignments of the three serotypes used in the study (Supplementary Fig. 3). We found that, overall, Regions 1–5 tended to be in conserved regions of the NS1 protein. In Region 1, to which responses were more potent in DENV1 and DENV3, we found that these serotypes shared 7 aa that were different from DENV2. Similarly, serum reactivity to Region 4 was only observed in DENV3 arrays, and there were 5 aa differences from DENV1 and DENV2.

We had observed increased overall magnitude of antibody responses in the NS1 vaccination sera compared to natural infection in Figure 1C. We next asked whether this increase in magnitude was a result of increased breadth (i.e., greater number of regions recognized). In comparing the responses to the five immunodominant regions identified in the NS1+MA vaccinated mice to those elicited by DENV2 infection, we found that while Regions 2, 4 and 5 were strongly targeted in both groups, DENV2-infected mice generated weak responses to Region 1 and no detectable responses to Region 3 (Fig. 2). Furthermore, no additional epitopes were detected in the DENV2-infected mice that were not present in the NS1-vaccinated mice.

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Mapping of peptide regions on the NS1 structure

The five regions of antibody reactivity were mapped to the molecular structure of the NS1 dimer (48), as shown in Fig. 3. Region 1 is exposed on the dimer surface and near the membrane-binding "greasy-finger" domain of NS1. The immunodominant Region 2 is in the surface-exposed disordered distal tip of the wing domain. Responses to four peptides in Region 2 were cross-reactive in the NS1+MA group across all three DENV serotypes strains and were also observed in the NS1+SCpG vaccinated mice (Supplementary Fig. 2). Region 3 mapped to the greasy finger domain of NS1 that in the dimeric form of NS1 is thought to interact with the cell membrane (49). Region 4 is in the surfaced exposed "spaghetti loop" region above the β̃ladder (48). Region 5 is on the outer surface of the dimer perpendicular to the wing domain. In conclusion, all five regions of antibody reactivity are surface exposed in the dimeric form of NS1.

NS1 antibody profiles of human subjects naturally infected with DENV2 reveal significant differences across timepoints

We used the same DENV peptide microarray to profile human antibody responses in plasma obtained from children naturally infected with DENV2 or DENV3 collected in the Nicaraguan Hospital-based Dengue Study. We profiled the antibody responses of 10 subjects with DF, 7 subjects with DHF, and 3 subjects with DSS. Sixteen of the subjects had samples available during the acute phase of infection, and all 20 had convalescent-phase samples as well as 12-month samples. As expected, we found overall stronger total anti-peptide antibody responses to DENV2 at convalescence compared to the acute phase (p=0.0005, Fig. 4A). The responses at the 12-month timepoint were lower than the convalescent phase, and this difference trended towards significance p=0.0547, Fig. 4A). The responses at the 12-month timepoint were higher than in the acute phase, but not significantly so (p=0.074). We then analyzed the differences in magnitude stratified by clinical phenotype (DF, DHF, and DSS) (Fig. 4B–D). Analysis of response magnitude and breadth was conducted separately for primary (n=4) and secondary (n=16) infections. We observed that in primary infections in both DF and DHF groups, the magnitude of responses at the 12-month timepoint did not wane and the response breadth was increased compared to the responses at the convalescent timepoint (Supplemental Fig. 4B–C and data not shown). However, in secondary infections, this was the case only in the DHF group.

Human subjects infected with DENV2 generate responses to Regions 2 and 5

To identify the epitopes targeted during the convalescent phase, we used the same statistical methodology as used in the mouse vaccination studies to compare the responses of the acute vs. convalescent timepoints. We found that the responses to Region 2 peptides were observed almost solely in the convalescent group (Table 1, Fig. 5A–C), while the response to this region almost completely waned at the 12-month timepoint (Fig. 5 and Supplementary Fig 4A). Comparing the acute response to Region 2 peptides across clinical phenotypes, we observed no responses in DF patients, weak responses in DHF patients and strong responses in DSS patients, similar to convalescent responses (Fig. 5). Responses to Region 5 were overall weaker than Region 2 and also waned at the 12-month timepoint.

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DISCUSSION

In this study, we found that mice vaccinated with recombinant DENV2 NS1 in combination with MPLA+AddaVax or SAS+CpG generated robust antibody responses to NS1 that provided protection from a lethal DENV2 challenge, whereas mice vaccinated with NS1 in combination with alum generated weak antibody responses that were not protective. To further characterize the antibody repertoire generated by vaccination and infection, we used peptide microarrays. We profiled the responses of vaccinated mice and compared these responses to mice infected with a non-lethal dose of DENV2. Overall, we found that mice vaccinated with NS1 combined with different adjuvants generated distinct NS1 antibody profiles that were different from those induced by natural infection. By comparing the responses of the alum group to the MA group, we identified 5 regions of DENV NS1 with significant levels of antibody reactivity in the MA group. Responses to some of these regions were cross-reactive across all three DENV serotypes tested, whereas others were only partially cross-reactive, or only reacted significantly to a single DENV serotype. Furthermore, antibody responses to two of these regions were only observed in mice vaccinated with DENV2 NS1. Additionally, we profiled the antibody responses to NS1 of humans naturally infected with DENV2 or DENV3 using plasma samples collected longitudinally at acute, convalescent and 12-month timepoints. We found that the responses across time differed in magnitude, with the strongest responses at the convalescent phase. When comparing the acute and convalescent responses we found that the reactivity to Region 2 was significantly targeted in the convalescent phase.

A fundamental aspect of this work is the comparison of antibody responses to NS1 following NS1 vaccination with antibody responses to DENV infection. We tested sera collected from DENV2 NS1-vaccinated mice, DENV2-infected mice, and DENV2- and DENV3-infected humans. Region 2, spanning aa 101–135, was immunodominant, with reactivity across all three DENV serotypes. This high reactivity of four adjacent peptides across the different serotypes may suggest that multiple epitopes are being detected within Region 2. Because the reactivity was independently found in both vaccinated and infected mice as well as DENV-infected humans, the data support the use of the mouse model for analyzing NS1-specific immune responses. Region 2 mapped in the structure to the “wing” domain that has been suggested to be exposed in both the dimeric and hexameric forms of NS1 (48, 50). Our data identifying this crucial region as immunodominant is in agreement with previous work that found antibody reactivity to this region in both mice and humans (23, 51–53). Region 5 contains aa 296–335, and part of this sequence was independently found to be reactive in NS1-vaccinated mice, infected mice and convalescent sera in humans.

We found differential serotype cross-reactivity to each of the five regions identified in the vaccinated mice. While four of the five regions were immunodominant in DENV2, Region 4 (aa 231–255) was only recognized in DENV3. In addition, responses to Regions 3 and 5 were only significant in DENV2. There are several possible reasons for these observed differences in cross-reactivity profile. First, the lack of cross-reactivity may be due to differences in the amino acids sequence conservation of these regions (Supplementary Figure 3). Second, the differences in serotype binding may reflect inherent specificity of the

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antibodies due to differences in their binding affinity. Third, they may also result from differences in the peptide attachment to the microarrays, since different amino acids have different binding propensities to the hydrogel surface that may affect the peptide orientation on the array.

Several of the regions with peptide reactivity in the NS1-immunized mice were similar to those recognized by infected mice and humans, which supports the idea of eliciting protective epitopes from vaccination that are similar to infection. However, additional epitope reactivity was found only with vaccination. Regions 1 and 3 are located along the more hydrophobic region of the NS1 dimer, which faces in towards the lipid core of the hexamer and in the cell-associated dimeric form of NS1 is thought to appose the cell's plasma membrane (48, 50). Therefore, antibodies to these regions could block the formation of the NS1 hexamer and/or inhibit NS1 binding to cells, thus potentially inhibiting the pathogenic effects of NS1. In fact, we previously found that passive transfer of polyclonal sera from NS1-vaccinated mice as well as a monoclonal antibody (1H7.4) directed to Region 1 (54) were able to completely protect mice against lethal challenge with DENV2 plus DENV2 NS1 (22). Therefore, immunization with recombinant NS1 may generate antibodies to regions that are not usually exposed in natural infection and could provide additional protection against a lethal DENV infection. Furthermore, DENV2 NS1 vaccination elicited serotype-specific and cross-reactive responses that could potentially protect against all three DENV serotypes. This is consistent with previous data, where we observed partial protection against DENV2 infection after immunization with DENV1 NS1, DENV3 NS1 or DENV4 NS1 protein (22).

When comparing the longitudinal responses to the NS1 peptide array in DENV-infected human populations, we found that overall, responses were higher in the convalescent phase than the acute phase. In primary infections, the magnitude of responses at the 12-month timepoint did not wane, and we observed an increase in the response breadth, i.e. the number of targeted antigens. However, in secondary infections, we found that only responses in the DHF group retained magnitude and increased breadth. Due to the limited number of human samples tested in this study, additional studies are required to validate these findings.

The peptide microarrays used in this study allowed us to obtain high-resolution epitope mapping of vaccine- and natural infection-induced antibody responses. We have previously shown that such arrays can be used to profile vaccine-induced responses of mice vaccinated with influenza vaccines (33, 34). We further found that immunodominant responses detected by our peptide arrays were in surface-exposed, functionally important regions of the NS1 protein. This is in agreement with our previous findings in an influenza vaccination study in mice, which reported that the immunodominant responses to the hemagglutinin (HA) protein were all surface-exposed on the HA trimer structure (34). Here, by using overlapping 20-mer peptides with 15-aa overlap, we identified specific regions in DENV NS1 that are epitope hotspots. Coupled with a rigorous statistical approach that identified immunodominant regions in each of the DENV serotypes independently, we were able to identify a small number of regions to which NS1 vaccines and natural DENV infection generated antibody responses. These findings further highlight the feasibility of using these peptide microarrays for antibody profiling in studies of dengue vaccines and natural infections.

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One limitation of our approach is the use of linear peptides that only capture part of the antibody repertoire; therefore, we may not have detected conformational or discontinuous epitopes. Nonetheless, we found that all of the identified peptides mapped to exposed regions of the NS1 protein (48, 50), some of which have been previously identified as antibody binding sites and regions of interest (23, 48, 53). There are several additional limitations of our current study. Since our peptide arrays were initially designed to map human responses from Nicaraguan patients, where there is very little circulation of DENV4, we only analyzed responses to 3 out of 4 globally circulating DENV serotypes. The number of DSS patients in this study was very small, and additional studies are required on a larger set of patients to address potential differences in peptide reactivity according to disease severity. Finally, we only mapped the responses of two SCpG NS1-vaccinated mice and thus could not statistically evaluate the responses to vaccination with this adjuvant combination.

Despite these limitations, we were able to identify a small number of immunodominant regions targeted following vaccination and natural infection, which were in functionally important locations on the NS1 protein both in its dimeric and hexameric forms. Combined with our previous data showing that passive transfer of polyclonal sera from NS1-immunized mice protected mice against DENV NS1-mediated lethal disease (22), we suggest that antibody responses to the regions identified here may play a role in protection and that they should be further studied in both animal models and humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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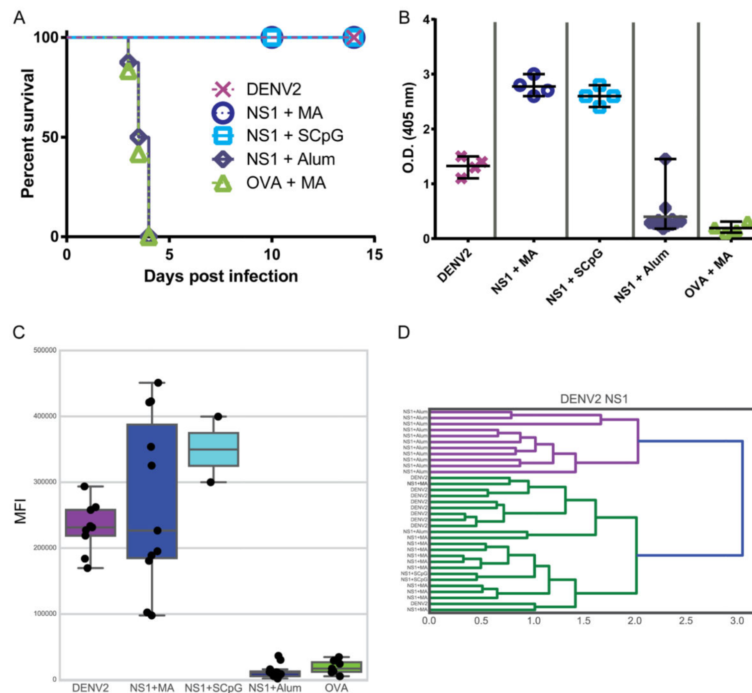

FIGURE 1.

Protective immunization with DENV NS1 results in the robust production of NS1-specific antibodies. (**A**) Survival curve of mice immunized with NS1 in combination with different adjuvants after lethal DENV2 challenge. Six- to eight-week old A129 mice (n=8 per group) were immunized i.p. with 20 µg recombinant DENV2 NS1 combined with MPLA + AddaVax (MA), Sigma Adjuvant System (SAS) + CpG (SCpG) or alum, or 20 ug OVA combined with MA on day 0, boosted on day 14 and day 42, and challenged i.v. on day 56 with a lethal dose of 10^7 pfu of DENV2 D220. In parallel, mice were infected with a sublethal dose (10^5 pfu) of DENV2 strain PL046 that provided protection from a lethal infection. $p<0.0001$ between NS1+MA or NS1+SCpG and OVA by Mantel-Cox log rank test. (**B**) NS1 IgG ELISA using mouse serum collected on day 49 after NS1 or OVA vaccination or sublethal DENV2 infection. Total anti-NS1 IgG was measured by direct ELISA, with sNS1 coated at 0.25 ug/mL and serum samples diluted 1:100. Biotinylated anti-mouse IgG secondary Ab was used for detection. **(C)** Boxplots of overall magnitude of the DENV2 NS1 response to the peptide array in each experimental group. The overall magnitude is defined by the sum of magnitudes to all NS1 peptides (see Methods for details). Horizontal bars represent the median response of each group. Box limits represent the 25th and 75th percentiles, and the whiskers denote the data range. Individual responses are plotted as filled circles. Responses above the whiskers are outliers. **(D)** Clustering dendrogram of all mouse samples generated using complete-linkage clustering over the DENV2 NS1 antibody profiles of each mouse (see Methods for details). Mice from different experimental groups were separated into distinct clusters. The MA-and SCpG-vaccinated mice cluster closer to the DENV2 naturally infected mice than to the alum-vaccinated mice.

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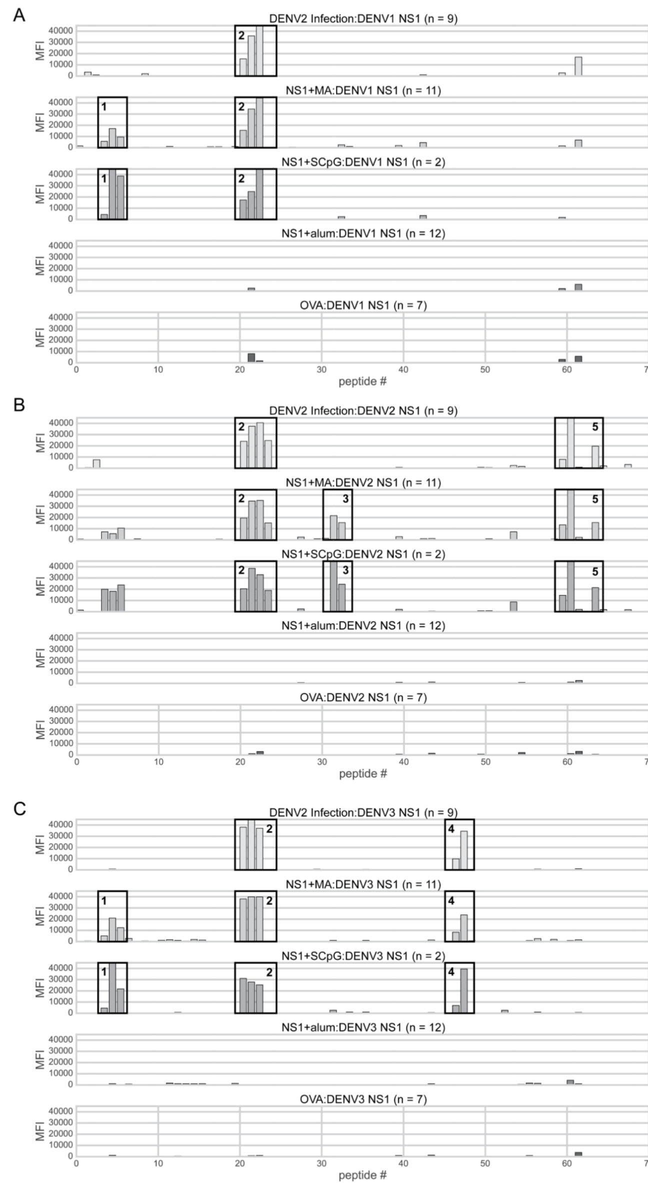

FIGURE 2.

DENV NS1 antibody profiles of NS1-vaccinated mice. Antibody reactivity to peptides derived from NS1 from: **(A)** DENV1; **(B)** DENV2; and **(C)** DENV3. Individual bars represent the average response of each experimental group to a specific NS1 peptide (n=68). The height of each bar represents the mean fluorescent intensity (MFI). For each serotype, responses of 5 groups are plotted: DENV2 infection – mice infected sub-lethally with PL046 (positive control, n=9); NS1+MA – mice vaccinated with NS1 adjuvanted with MPLA + AddaVax (n=11); NS1+SCpG – mice vaccinated with NS1 adjuvanted with SAS + CpG (n=2); NS1+alum – mice vaccinated with NS1 ajduvanted with alum (n=12); OVA – mice vaccinated with OVA adjuvanted with MPLA + AddaVax (negative control, n=7). The five dominant regions statistically identified are highlighted by the numbered rectangles across all serotypes and groups. Robust responses to Region 2 are observed in the DENV-infected,

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NS1+MA and NS1+SCpG groups across all three DENV serotypes. Responses to Regions 1 and 3 were observed only in NS1+ MA and NS1+SCpG vaccinated mice, and not in DENV-infected mice.

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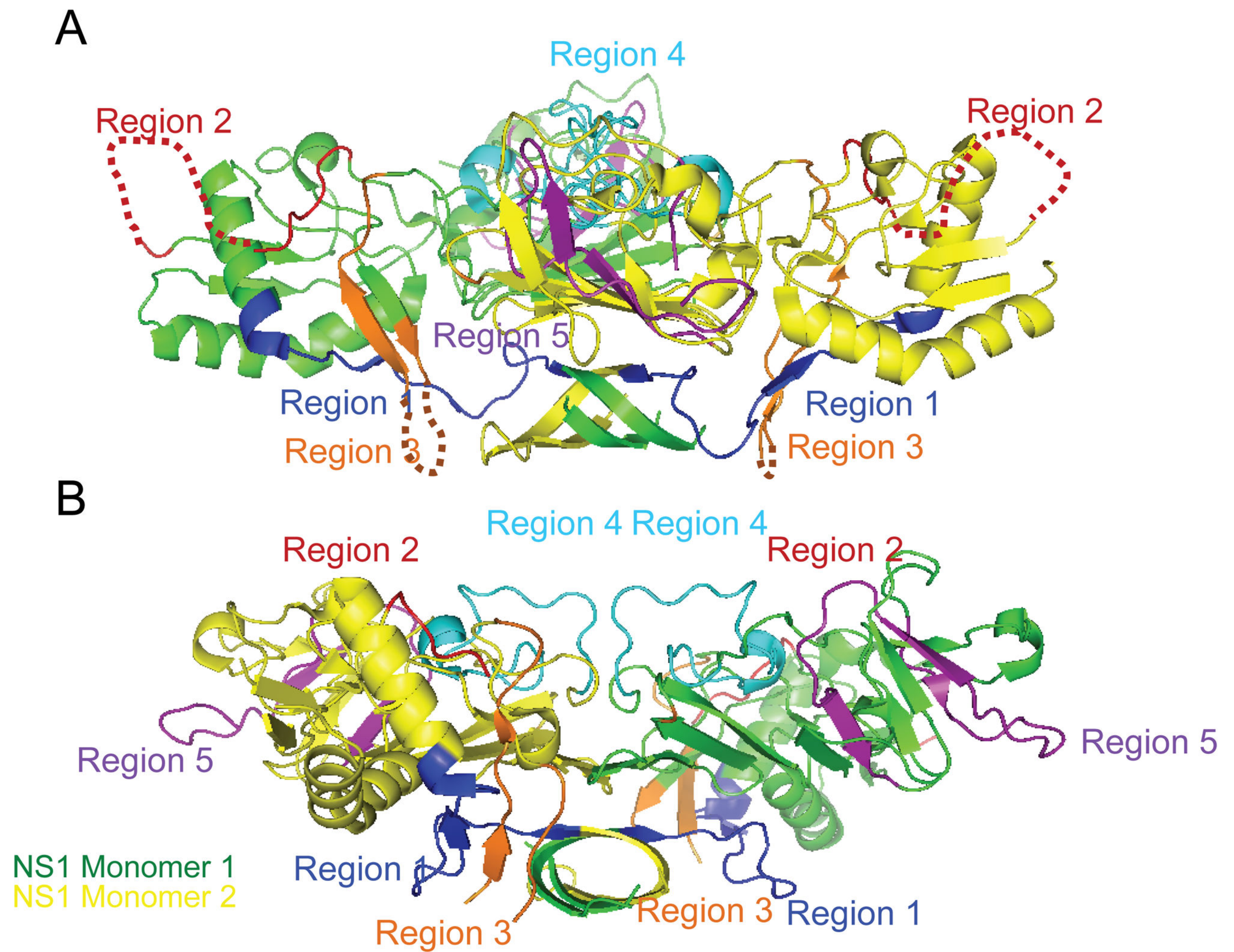

FIGURE 3.

Structural model of NS1 showing key epitopes recognized by sera from NS1-immunized mice. NS1 dimer structures highlighting the 5 peptide regions of antibody reactivity, with monomer 1 in green and monomer 2 in yellow and the peptide regions colored as follows: blue, Region 1, aa21–40; red, Region 2, aa101–130; orange, Region 3, aa156–175; light blue, Region 4, aa231–255; purple, Region 5, aa296–325. **(A)** Perpendicular view of NS1 from the end of the β-ladder. The mobile "greasy finger" and the disordered tip of the wing domain are indicated by dotted lines. **(B)** Perpendicular view of NS1 from the edge of the β-ladder. PDB 4O6B (48).

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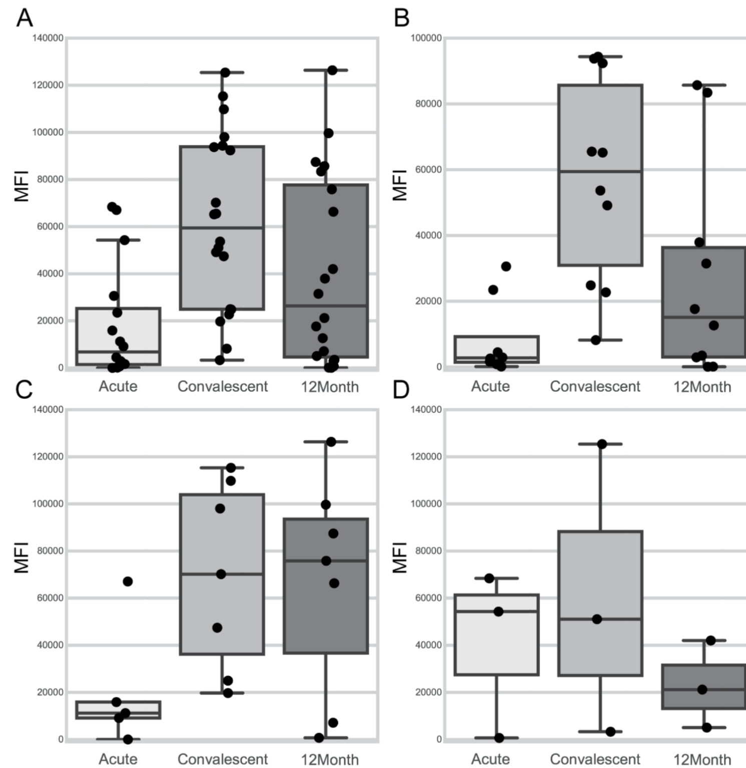

FIGURE 4.
DENV2 NS1 magnitude (pooled reactivity) boxplots of samples from human subjects naturally infected with DENV2 or DENV3 across timepoints. Horizontal bars represent the median response of each group. Box limits represent the 25th and 75th percentiles, and the whiskers denote the data range. Individual responses are plotted as filled circles. Responses above the whiskers are outliers. **(A)** Pooled reactivity to NS1 peptides (magnitude) is increased in convalescent sera and declines in samples collected 12 months post-illness. **(B–D)** Boxplots of the magnitude of the NS1-specific response across time stratified by clinical phenotype: **(B)** Dengue Fever, **(C)** Dengue Hemorrhagic Fever, and **(D)** Dengue Shock Syndrome.

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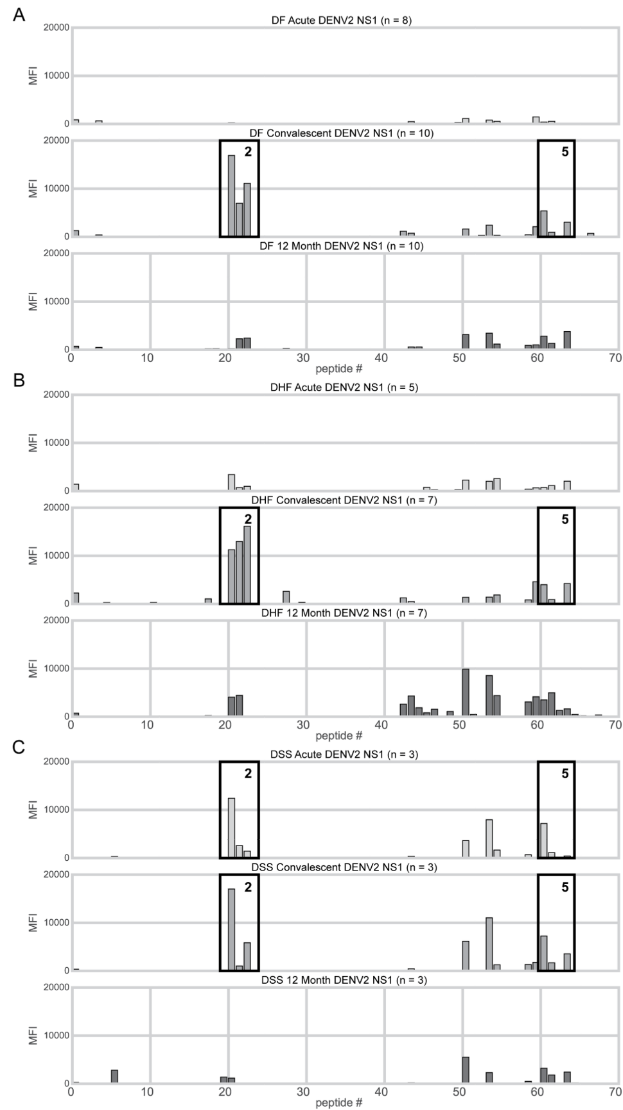

FIGURE 5.

Antibody responses to DENV2 NS1 over time stratified by clinical phenotype. In each plot, the bars represent the average response of subjects with a given clinical phenotype. Responses are plotted at the acute, convalescent and 12-month timepoints. The number of subjects in each group and timepoint is indicated in parentheses. **(A)** DF responses; **(B)** DHF responses; **(C)** DSS responses. Regions 2 and 5 identified in vaccinated mice were independently identified in the human natural infection responses.

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Table 1

NS1 Regions identified with peptide arrays probed with mouse and human sera.

Region	NS1 peptide number	NS1 aa start position	Mouse studies, p-values Alum vs. MA (Bonferonni adjusted)#			Human studies, p-values Acute vs. Convalescent (Bonferonni adjusted)#		
			DENV1	DENV2	DENV3	DENV1	DENV2	DENV3
1	5	21	0.027400	*	0.008076	*	*	*
2	21	101	0.027460	0.000007	0.000004	*	0.028623	0.001523
	22	106	0.002019	0.000007	0.000004	*	0.265688	*
	23	111	0.000058	0.000007	0.000004	0.009091	0.000248	*
	24	116	*	0.012115	*	*	*	*
3	32	156	*	0.041190	*	*	*	*
4	47	231	*	*	0.027460	*	*	*
	48	236	*	*	0.000058	*	*	*
5	60	296	*	0.012115	*	*	*	*
	61	301	*	0.000087	*	*	0.335293	*
	64	316	*	0.000087	*	*	*	*

#To determine significant differences in reactivity between groups for each peptide of a specific DENV serotype, we compared total MFI between groups, adjusting for multiplicity using Bonferroni as described in the Methods.

*Peptide antigens that were filtered out using a label-blind filtering approach in which peptides with an overall response rate of less than 20% were not tested (see Methods for details).