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Protection from Secondary Dengue Virus Infection in a Mouse Model Reveals the Role of Serotype Cross-Reactive B and T Cells

Simona Zompi,* Brian H. Santich,* P. Robert Beatty,*[†] and Eva Harris*

The four dengue virus (DENV) serotypes cause dengue fever and dengue hemorrhagic fever/dengue shock syndrome. Although severe disease has been associated with heterotypic secondary DENV infection, most secondary DENV infections are asymptomatic or result in classic DF. The role of cross-reactive immunity in mediating cross-protection against secondary heterotypic DENV infection is not well understood. DENV infection of IFN- α/β and IFN- γ receptor-deficient (AG129) mice reproduces key features of human disease. We previously demonstrated a role in cross-protection for pre-existing cross-reactive Abs, maintained by long-lived plasma cells. In this study, we use a sequential infection model, infecting AG129 mice with DENV-1, followed by DENV-2 6–8 wk later. We find that increased DENV-specific avidity during acute secondary heterotypic infection is mediated by cross-reactive memory B cells, as evidenced by increased numbers of DENV-1-specific cells by ELISPOT and higher avidity against DENV-1 of supernatants from polyclonally stimulated splenocytes isolated from mice experiencing secondary DENV-2 infection. However, increased DENV-specific avidity is not associated with increased DENV-specific neutralization, which appears to be mediated by naive B cells. Adoptive transfer of DENV-1-immune B and T cells into naive mice prior to secondary DENV-2 infection delayed mortality. Mice depleted of T cells developed signs of disease, but recovered after secondary DENV infection. Overall, we found that protective cross-reactive Abs are secreted by both long-lived plasma cells and memory B cells and that both cross-reactive B cells and T cells provide protection against a secondary heterotypic DENV infection. Understanding the protective immunity that develops naturally against DENV infection may help design future vaccines. *The Journal of Immunology*, 2012, 188: 404–416.

Dengue, caused by four dengue virus serotypes (DENV-1–4), is the most prevalent mosquito-borne viral disease in humans. Clinical disease ranges from asymptomatic infection and classic dengue fever (DF) to more severe forms, dengue hemorrhagic fever/dengue shock syndrome. Approximately 40 million cases of dengue and 250,000–450,000 dengue hemorrhagic fever/dengue shock syndrome cases are estimated to occur each year (1). Whereas previous infection with one DENV serotype induces long-lasting protective humoral and T cell responses against the same serotype, reinfection with a different serotype has been associated with more severe disease (2–6). Cross-reactive Abs acting through Ab-dependent enhancement (ADE) (7–9), as well as cross-reactive T cells (6, 10–13), have been implicated in increased disease severity after secondary (2°) infection. However, most 2° infections are asymptomatic or result

in classic DF, indicating that cross-reactive immunity can be protective (3). Cross-reactive Abs have been correlated with less severe disease in humans (14, 15), and we have previously shown in a mouse model of dengue that passive transfer of cross-reactive Abs resulted in reduced viral load in multiple organs after a subsequent nonlethal heterotypic DENV infection (16).

In terms of the cellular immune response, it is still unclear what the specific roles of memory B cells and memory T cells are in DENV cross-protection. Different B cell compartments can be identified according to their phenotype (17). Affinity-matured memory B cells persist as non-Ab-secreting cells, but maintain their Ig as membrane bound and are the precursors of the rapid cellular response to Ag recall (17). Upon Ag recall, memory B cells differentiate into short-lived plasma cells (PCs) and long-lived PCs (LLPCs). LLPCs are terminally differentiated, nondividing cells, which home to the bone marrow and are responsible for the long-term humoral response (17). Both long-lasting specific Ab responses, attributed to LLPCs, and long-lived memory B cells contribute to long-term protective immunity (18, 19). Maintenance of LLPCs has been shown to be independent of memory B cells (20), indicating that LLPCs are sufficiently long-lived to sustain Ab titers for a long period of time. Moreover, in humans, a lack of linear correlation between tetanus toxin-specific memory B cells and serum titers of tetanus toxin-specific IgG over time (18) indicates that memory B cells and LLPCs represent independent forms of immunological memory.

We have developed an IFN- α/β and IFN- γ receptor-deficient (AG129) mouse model of dengue that reproduces both ADE and Ab-mediated protection (7, 16, 21). DENV infection of AG129 mice recapitulates key features of human disease, including vascular leak, low platelet counts, and increased levels of serum cytokines such as IL-10 and TNF- α . Tropism studies identified

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Abbreviations used in this article: ADE, Ab-dependent enhancement; ASC, Ab-secreting cell; CP, cyclophosphamide; DC, dendritic cell; DENV, dengue virus; DF, dengue fever; LLPC, long-lived plasma cell; NT₅₀, 50% neutralization titer; PC, plasma cell; p.i., postinfection; SIGN, specific intercellular adhesion molecule-3-grabbing nonintegrin; WT, wild-type.

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DENV in relevant tissues and cells, such as dendritic cells (DCs) and macrophages (22). All four DENV serotypes replicate efficiently in AG129 mice after administration of DENV by either a s.c. or i.v. route. The generation of a more virulent and lethal DENV strain, DENV-2 D2S10, allowed us to study pathogenesis of severe disease in vivo (7, 23, 24). The two mutations that differentiate D2S10 from the parental PL046 DENV-2 strain, N124D and K128E in the virus envelope protein, decrease heparan sulfate binding and consequently reduce clearance of the virus, thus increasing viremia and resulting in the lethal disease phenotype (25). AG129 mice infected with high doses of D2S10 develop signs of vascular leak, low platelet counts, and high levels of serum cytokines, including increased IL-10 and TNF- α , and exhibit mortality within 4–5 d due to a nonparalytic syndrome (23). We previously demonstrated a role for the cross-reactive cellular immune response and for pre-existing cross-reactive Abs during 2° DENV using cyclophosphamide (CP). CP is an alkylating agent and immunosuppressive drug that primarily affects proliferating cells (26, 27) and thus eliminates the proliferating cellular immune response during a 2° DENV infection. CP-treated naive mice exhibited mortality from a primary (1°) DENV-2 D2S10 sublethal infection 3–4 d earlier than DENV-1-immune mice treated with CP (21). Naive mice have no pre-existing anti-DENV Abs, as compared with DENV-1-immune mice, which have pre-existing cross-reactive Abs. Thus, this experiment demonstrates the contribution of pre-existing cross-reactive Abs to protection during the early phase of DENV infection (21). These data are consistent with experiments that showed that passive transfer of heterotypic serum prior to DENV infection reduces viral load in several tissues (16). The fact that DENV-1-immune mice treated with CP prior to a 2° DENV-2 D2S10 sublethal infection experienced lethal disease (21) demonstrates a role for the cellular immune response in protection. Morbidity and mortality in these mice correlated with increased viremia 24 h prior to death as compared with CP-untreated controls (21). In addition, adoptive transfer of immune splenocytes into naive mice prior to infection demonstrated that the cellular immune compartment contributes to the control of DENV viral load as measured in several tissues during a 2° heterotypic DENV infection (16). The T cell response, and more specifically homotypic anti-DENV CD8⁺ T cells, has been reported to protect mice against a 1° DENV infection (28). CD4⁺ T cells were found to be dispensable for the induction of DENV-specific CD8⁺ T cells and Ab response during a 1° infection, but were found to have cytolytic activity in vivo against DENV-infected cells and to increase the cytolytic activity of CD8⁺ T cells after vaccination, indicating a role for these cells during a 2° response (29). Together these data demonstrate a role for CD4⁺ and CD8⁺ T cells in homotypic protection. However, it is still not clear whether cross-reactive T cells can be protective during a 2° heterotypic DENV infection.

In this study, we first characterized the murine B cell and humoral response during 2° heterotypic DENV-2 infection by measuring B cell proliferation, DENV-specific neutralization capacity, and DENV-specific avidity of serum and supernatants of polyclonally stimulated splenocytes using a modified urea-wash ELISA. We also measured the number of DENV-specific memory B cells and PCs by ELISPOT. Subsequently, we dissected the role of the cellular immune response by adoptively transferring different cellular subsets and by using mAbs to deplete distinct immune cell subsets in vivo. In summary, we show that pre-existing cross-reactive Abs and Abs newly secreted by cross-reactive memory B cells participate in cross-protection against a 2° heterotypic DENV infection. In addition, we demonstrate that cross-reactive B and T cells as well as innate immune cells all

participate in protection against a lethal 2° heterotypic DENV infection and that T cells are necessary for full protection in this model.

Materials and Methods

Viruses and cell lines

DENV was propagated in *Aedes albopictus* cell line C6/36 (gift of P. Young, University of Queensland) in M199 medium (Invitrogen) with 10% FBS (Denville Scientific) at 28°C. Cell supernatants were collected on days 5, 6, 7, and 8 postinfection (p.i.) and either frozen at –80°C directly or after concentration. Concentrated virus was prepared by either ultracentrifugation of the supernatants (26,000 \times g for 2 h at 4°C) or centrifugation through Amicon filters (Millipore; 50 kDa, 3,250 \times g for 20 min at 4°C). DENV-2 strain D2S10 (passage 4) is a peripherally passaged mouse-adapted strain derived in our laboratory from the parental DENV-2 PL046 Taiwanese strain, as described elsewhere (23). DENV-1 strain 98J (passage 7) was isolated in our laboratory from a patient from Guyana in 1998 (30). Virus titers were obtained by plaque assay on baby hamster kidney cells (BHK21, clone 15), as previously described (31). U937-DC-specific intercellular adhesion molecule-3-grabbing nonintegrin (SIGN) cells (gift of A. de Silva, University of North Carolina, Chapel Hill) were grown in RPMI 1640 (Invitrogen) with 2% FBS, at 37°C in 5% CO₂ for use in neutralization assays (32).

Infection of mice

AG129 mice lacking receptors for IFN- α/β and IFN- γ were obtained from M. Aguet (Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland) and bred in the University of California Berkeley Animal Facility. All experimental procedures were preapproved by the University of California Berkeley Animal Care and Use Committee and were performed according to the guidelines of the University of California Berkeley Animal Care and Use Committee. Experiments were initiated with 6- to 8-wk-old mice. The s.c. injections, in a total volume of 200 μ l, were performed under the skin of the ventral hindlimbs; tail vein i.v. injections were performed using a total volume of 100 μ l; and i.p. injections, using 100–200 μ l, were performed through the lower left abdominal wall. For 1° infections, mice were infected s.c. with 10⁵ PFU DENV-2 D2S10. For 2° infections, mice were infected first with 10⁵ PFU DENV-1 98J s.c., and then, 6–8 wk later, were infected a second time with 10⁷ PFU DENV-2 D2S10 i.v. Mock-infected control mice (post-1°/pre-2°) were injected with uninfected C6/36 supernatant. A129 mice, lacking the IFN- γ receptor, were obtained from H. Virgin (Washington University School of Medicine, St. Louis, MO), and wild-type (WT) 129 mice were obtained from Taconic Farms. For adoptive transfer experiments (see below), AG129, A129, and WT129 mice were infected s.c. with 10⁵ PFU DENV-1 98J, and then, 6–8 wk later, spleens were collected, pooled, and used to isolate B and T cells. To assess morbidity associated with DENV infection, mice were scored on a scale from 1 to 5, as follows: 1) healthy; 2) displaying mild signs of lethargy, some fur ruffling, and no hunched posture; 3) displaying mild signs of lethargy, fur ruffling, and hunched posture; 4) displaying increased signs of lethargy and limited mobility, fur ruffling, and hunched posture; and 5) moribund. Mice were euthanized when moribund per University of California Berkeley animal care guidelines.

In vivo T cell depletion

AG129 mice were infected s.c. with 10⁵ PFU DENV-1 98J, and 6–8 wk later on day 0 were infected i.v. with 10⁷ PFU DENV-2 D2S10. On days –1, 0, 1, and 2 p.i. mice were treated i.p. with 200 μ g anti-CD4 Ab (clone GK1.5, IgG2b), and on days –1, 0, 1, 2, and 7 p.i. were treated i.p. with 400 μ g anti-CD8 Ab (clone 2.43, IgG2a). Control mice received either isotype control Ab (IgG2a for depletions of CD8⁺ T cells and IgG2b for depletions of CD4⁺ T cells) or PBS i.p. Morbidity and mortality were monitored for 10 d. Mice were euthanized at day 10 post-2° infection, and spleen cells were harvested to test the efficacy of T cell depletion by flow cytometry using anti-CD3 PECy5 (clone 17A2), anti-CD4 PECy7 (RM4-5), and anti-CD8 FITC Abs (clone 53-6.7).

In vivo B cell depletion

AG129 mice were infected s.c. with 10⁵ PFU DENV-1 98J, and 6–8 wk later on day 0 were infected i.v. with 10⁷ PFU DENV-2 D2S10. On day 8, mice were treated i.v. with 250 μ g anti-CD20 Ab (clone 18B12, IgG2a; Biogen Idec). Control mice received either isotype control Ab (IgG2a) or PBS i.v. Morbidity and mortality were monitored for 10 d. Whole blood collected by eye bleed prior to 2° infection was used to determine the

efficacy of B cell depletion. Flow cytometry was performed on whole blood cells using anti-CD19 PE, anti-B220 PECy7, and anti-IgD peripheral blood Abs 8 d after depletion.

Adoptive transfer of T cells

A129 mice were infected s.c. with 10^5 PFU DENV-1 98J, and, 6–8 wk later, spleens were harvested and pooled. CD4⁺ and CD8⁺ T cells were isolated using MACS bead CD4⁺ and CD8⁺ T cell negative selection isolation kit (Miltenyi Biotec). The purity of cells was assessed by flow cytometry using anti-CD3 PECy5, anti-CD4 PECy7, and anti-CD8 FITC Abs. Isolated cells (2×10^5) were adoptively transferred i.v. into naive AG129 mice, and, 24 h later, mice were infected i.v. with 10^7 PFU DENV-2 D2S10. Mice were monitored for morbidity and mortality for 10 d.

Adoptive transfer of B cells

AG129 or WT129 mice were infected s.c. with 10^5 PFU DENV-1 98J, and, 6–8 wk later, spleens were harvested and pooled. Total B cells were isolated using a MACS bead B cell negative selection isolation kit (Miltenyi Biotec). Cell purity was evaluated by flow cytometry using anti-CD19 PE and anti-B220 PECy7 Abs. Isolated cells (30×10^6) were adoptively transferred i.v. into AG129 naive mice, and, 24 h later, mice were infected i.v. with 10^7 PFU DENV-2 D2S10. Mice were monitored for morbidity and mortality for 10 d.

Flow cytometry

On days 3, 6, and 9 after 2° DENV infection, spleens were collected, and single-cell suspensions were prepared for flow cytometry. Cells were stained with anti-IgD peripheral blood, anti-B220 PECy7, anti-CD79b FITC, and anti-CD138 allophycocyanin. Proliferating memory B cells in the spleen were quantified using anti-Ki-67 (BD Pharmingen) (33). Results were analyzed using FlowJo software, version 7.2.5 (Tree Star Software).

ELISPOT

To quantify the number of DENV-specific B cells and PCs, total splenocytes or bone marrow cells were analyzed by ELISPOT ex vivo (reflecting the PC compartment producing Ab in vivo at the time of collection) or after in vitro stimulation (reflecting the memory B cell compartment present in vivo at the time of collection). For the bone marrow, two femurs per mouse were harvested. The cell count obtained from the two femurs was then multiplied by 7.9 to estimate the total number of bone marrow cells per mouse (34). For in vitro stimulation, splenocytes were polyclonally stimulated in vitro for 6 d with PWM extract (0.1 µg/ml; Sigma-Aldrich), CpG oligonucleotide (1 µg/ml), 1/10,000 fixed *Staphylococcus aureus* Cowan (Sigma-Aldrich), and LPS (10 µg/ml; Sigma-Aldrich) to generate Ab-secreting cells (ASCs) (35). For the ELISPOT, 96-well filter plates were coated with DENV-1 98J or DENV-2 D2S10 cellular Ag prepared from infected C6/36 cells. To detect total IgG-secreting cells, wells were coated with goat anti-mouse IgG (2.5 µg/ml; Jackson ImmunoResearch Laboratories). To prepare cellular Ag, C6/36 cells were infected with DENV-1 98J or DENV-2 D2S10 at a multiplicity of infection of 0.1, collected 7 d p.i., and submitted to seven cycles of freeze/thaw; cell lysates were then suspended in 1% Tween 20 and 50% glycerol and stored at -80°C . Uninfected cells were submitted to the same procedure to produce mock-infected control cellular Ag. Ag-coated plates were incubated for 5–6 h with Ab-producing cells to allow formation of Ab–Ag complexes. Cells were removed, and plates were washed and incubated with anti-mouse IgG HRP-conjugated Ab overnight (1/1000; Sigma-Aldrich), followed by substrate (amino-9 ethyl-carbazole; Sigma-Aldrich). Resulting spots, representing DENV-specific Ab-producing B cells or total IgG Ab-producing cells, were counted by visual inspection using an inverted microscope. The number of spots counted in the control wells, coated with mock-infected control cellular Ag, was subtracted from the number of spots counted in each test well coated with DENV-specific cellular Ag.

Neutralization assay

Serum was diluted using eight 4-fold dilutions, beginning at 1:20 to 1:327,680. A total of 6×10^2 PFU D2S10 in 30 µl was mixed with 10 µl each serum dilution and incubated for 45 min at 37°C . This viral titer was selected to satisfy the assumptions of the law of mass action (36). The virus/serum combinations were mixed with 5×10^5 cells and incubated for 18–24 h at 37°C . The cells were then fixed and stained both intracellularly for the virus using a fluorescently tagged DENV-specific mAb (4G2-Alexa488) and extracellularly for DC-SIGN (anti-CD209 PECy5, clone DCN46). The cells were processed by flow cytometry, and the percentage

of cells positive for DC-SIGN and infected with DENV was determined. To analyze the U937-DC-SIGN neutralization data, raw data were expressed in GraphPad as percentage of infection versus \log_{10} of the serum dilution. A sigmoidal dose-response curve with a variable slope was used to determine the serum dilution at which a 50% reduction in infection was observed compared with the no-serum control and was expressed as the 50% neutralization titer (NT_{50}).

Avidity assay

Serum avidity was measured using a modified ELISA protocol with urea washes (37, 38). Ninety-six-well plates were coated overnight with either DENV-1 or DENV-2 recombinant envelope protein (Hawaii Biotech) at 1 µg/ml, blocked, and incubated for 1 h with serum samples from 1° and 2° DENV infections. The plates were washed for 10 min with 6–9 M urea or PBS before adding biotin-conjugated goat anti-mouse IgG, streptavidin-alkaline phosphatase, and *p*-nitrophenyl phosphate (PnPP) substrate. OD was measured at 405 nm using KC Junior software. Background from the no-Ag-coated wells was subtracted from each sample. The percentage of IgG bound was calculated by dividing the OD after the urea wash by the OD after the PBS wash.

Statistical analysis

Kaplan–Meier survival curves were used to display mortality data, and log rank analyses were used to determine statistical significance between experimental groups. Nonparametric analyses were performed using the Mann–Whitney *U* test for unpaired comparisons. Calculations were performed in GraphPad Prism 5.0 software.

Results

DENV-1-immune mice are protected against a lethal heterotypic secondary DENV-2 infection

DENV-2 D2S10 resulted in 90% mortality in naive AG129 mice by day 5 p.i. by a characteristic vascular leak syndrome when administered at a high inoculum (10^7 PFU i.v.) (Fig. 1A) (7, 23). In contrast, mice infected first with DENV-1 98J (10^5 PFU s.c.) were completely protected against a 2° infection with a lethal dose of DENV-2 D2S10 6–8 wk after the 1° infection (Fig. 1A). DENV-1-immune mice did not show any signs of disease during 15 d of observation, nor any fluid accumulation in the visceral organs or increased viral load as measured by plaque assay (no detectable plaques in liver, bone marrow, small intestine, spleen, and WBCs) and by quantitative RT-PCR (in serum; data not shown) at day 3.5 post-2° infection with DENV-2 D2S10. In comparison, naive mice infected with a lethal dose of D2S10 displayed an increase in viral load in several tissues 12 h prior to death (day 3.5 p.i.) (7). In addition, platelet count, which decreases p.i. with a lethal dose of DENV-2 D2S10 in naive mice, and plasma levels of IL-10, which increase under these conditions (7), remained normal after a 2° infection with a lethal dose of DENV-2 D2S10 in DENV-1-immune mice (data not shown). Therefore, a 1° infection with DENV-1 provided complete protection from a 2° infection with DENV-2, with reduced viral load, no symptoms, and no resulting mortality.

We hypothesized that the protective immunity was mediated at least partially by memory B cells and/or LLPCs. Thus, 6–8 wk after a 1° DENV-1 infection, we performed an ELISPOT ex vivo using splenocytes and bone marrow cells (Fig. 1B) to measure DENV-specific ASC. The highest numbers of anti-DENV-1 ASC were found in the bone marrow. Representative photographs of the B cell ELISPOT are provided in Supplemental Fig. 1A. These cells are PCs, as ELISPOT assays performed ex vivo identify PCs, in contrast to ELISPOT assays performed after polyclonal in vitro stimulation, which identify memory B cells. As these PCs home to the bone marrow and are found several weeks p.i., they are most likely LLPCs (39). Thus, the long-term anti-DENV Ab response, mostly to DENV-1, is sustained by LLPCs. Of note, a substantial number of DENV-specific PCs was also found in the spleen ($886 \pm$

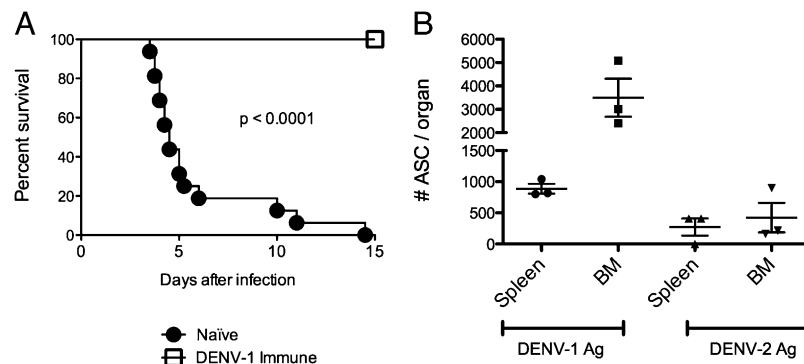


FIGURE 1. Protection of DENV-1-immune AG129 mice against a lethal 2° DENV-2 infection. *A*, Survival of AG129 mice after 1° and 2° DENV infection. AG129 mice were infected s.c. with 10^5 PFU DENV-1 98J (DENV-1 immune, $n = 14$) or injected s.c. with C6/36 cell supernatant (Naive, $n = 16$). Eight weeks p.i., the mice were infected i.v. with a lethal dose of DENV-2 D2S10 (10^7 PFU). Statistical analysis was performed using the Wilcoxon rank sum test. Survival of DENV-1-immune mice infected with DENV-2 D2S10 was significantly different from naive mice infected with DENV-2 D2S10. *B*, DENV-specific B cell ELISPOT after 1° DENV-1 infection. AG129 mice were infected s.c. with 10^5 PFU DENV-1 98J ($n = 3$). Six to 8 wk p.i., spleen and bone marrow were collected, and cell suspensions were prepared. Cells were tested ex vivo by ELISPOT using DENV-1 and DENV-2 cellular Ag to detect DENV-1- and DENV-2-specific PCs. The number of spots from control wells coated with mock Ag was subtracted from the number of spots counted in DENV-coated wells. Statistical analysis was performed using the Mann-Whitney U test. No significant difference in the number of ASCs was found between cells tested in the spleen and bone marrow.

78.4 serotype-specific PCs/spleen and 252 ± 136 serotype cross-reactive PCs/spleen (Fig. 1*B*). These cells are most probably semi-long-lived PCs that originate or migrate to the spleen after a 1° infection before becoming LLPCs (40).

B cells and CD8⁺ T cells proliferate upon a heterotypic secondary DENV infection

To characterize B cell and T cell immune responses after 2° DENV infection, spleens were collected 3, 6, and 9 d after heterotypic 2° infection, and B and T cell subsets were analyzed by flow cytometry. We detected increased proliferation of the memory B cell component (B220⁺, CD79b⁺, CD138⁻, IgD⁻) by day 6 post-2° infection in the spleen, using Ki-67 as a proliferation marker (post-1°/pre-2° = $0.54 \pm 0.11\%$ Ki-67⁺ versus mean day 6 p.i. = $1.52 \pm 0.42\%$ Ki-67⁺, $p = 0.09$) (Fig. 2*A*, 2*B*). Increased absolute numbers of memory B cells were detected in the spleen at day 9 post-2° infection (mean post-1°/pre-2° = $9.08 \times 10^5 \pm 4.22 \times 10^5$ cells/ml versus mean day 9 p.i. = $32.3 \times 10^5 \pm 4.20 \times 10^5$ cells/ml, $p = 0.008$) (Fig. 2*C*). The increase in absolute number of PCs (B220^{low/-}, CD79b⁺, CD138⁻, IgD⁻) in the spleen preceded the increase in absolute number of memory B cells, with a significant increase evident by day 6 p.i. (mean post-1°/pre-2° = $4.90 \times 10^5 \pm 2.14 \times 10^5$ cells/ml versus mean day 6 p.i. = $13.4 \times 10^5 \pm 0.81 \times 10^5$ cells/ml, $p = 0.049$) (Fig. 2*C*). Among T cells, CD4⁺ T cell numbers, although not significant, increased 2-fold after 2° heterotypic infection (mean post-1°/pre-2° = $9.88 \times 10^6 \pm 2.18 \times 10^6$ cells/ml versus mean day 9 p.i. = $18.9 \times 10^6 \pm 7.0 \times 10^6$ cells/ml, $p = 0.20$). We also detected a significant increase in absolute CD8⁺ T cell numbers by day 9 p.i. when compared with post-1°/pre-2° control mice (mean post-1°/pre-2° = $5.58 \times 10^6 \pm 1.03 \times 10^6$ cells/ml versus mean day 9 p.i. = $12.9 \times 10^6 \pm 4.13 \times 10^6$ cells/ml, $p = 0.04$) (Fig. 2*D*), with increased proliferation detectable by day 3 p.i. (mean post-1°/pre-2° = $0.86 \pm 0.35\%$ Ki-67⁺ versus mean day 3 p.i. = $4.80 \pm 2.31\%$ Ki-67⁺, $p = 0.38$).

The DENV-specific neutralization capacity of serum increases between days 3 and 6 postsecondary heterotypic infection and after day 6 postprimary infection

We used a U937-DC-SIGN flow cytometry-based assay to measure the neutralization titer of sera against DENV-2 on days 3, 6, and 9 after 2° heterotypic infection. As shown in Fig. 3*A*, the NT₅₀ against DENV-2 D2S10 significantly increased between days 3

and 6 after 2° infection ($p < 0.0001$). A similar increase in neutralization titer was also observed between days 3 and 6 after 1° infection with a sublethal dose of DENV-2 D2S10 (Fig. 3*B*). Thus, the increase in neutralization titer can be attributed to either cross-reactive memory B cells induced by the first DENV-1 infection or naive B cells responding to the DENV-2 D2S10 infection during the 2° heterotypic infection. Of note, we did not observe an increase in neutralization against DENV-1 on days 3, 6, and 9 after 2° heterotypic infection (Fig. 3*C*), suggesting that naive B cells are the cells responsible for the increase in neutralization against DENV-2 during the 2° heterotypic infection.

Cross-reactive memory B cells mediate an increase in DENV-specific serum avidity between days 3 and 6 postsecondary heterotypic DENV infection

We measured the avidity of Abs produced in serum 6–8 wk after a 1° DENV-1 infection (post-1°/pre-2°), reflecting the Abs produced by LLPCs, and compared it with the avidity of the Abs produced during the acute phase of a 2° heterotypic DENV-2 infection, using a modified ELISA involving urea washes (Fig. 4*A–D*). As expected, serum avidity decreased with increasing concentrations of urea (Fig. 4*A–D*). Based on these experiments, a single concentration of 7 M urea was then selected and used to compare avidity in the serum and in the supernatants of polyclonally stimulated splenocytes to DENV-1 versus DENV-2 Ag. The avidity of Abs in serum against both DENV-1 and DENV-2 increased significantly 6 d after 2° infection when compared with preinfection serum (Fig. 4*E*), and this increase in avidity was greater against DENV-1 than against DENV-2, suggesting an increase in cross-reactive Abs during the acute phase of the 2° heterotypic infection. Of note, avidity against DENV-1 or DENV-2 could not be measured 3, 6, or 9 d after a 1° infection as the level of Abs was below the limit of detection of the assay. Thus, the increase in serum avidity can be attributed to Abs secreted by memory B cells rather than activated naive B cells.

We then measured the avidity of supernatants from polyclonally stimulated splenocytes collected after 2° heterotypic DENV infection using 7 M urea in the ELISA washes. The Abs found in these supernatants are not secreted by PCs, as PCs do not usually survive polyclonal stimulation in cell culture (41). In addition, we measured the amount of Abs in the supernatant of splenocytes maintained in culture for 24 h only, which might contain Abs from

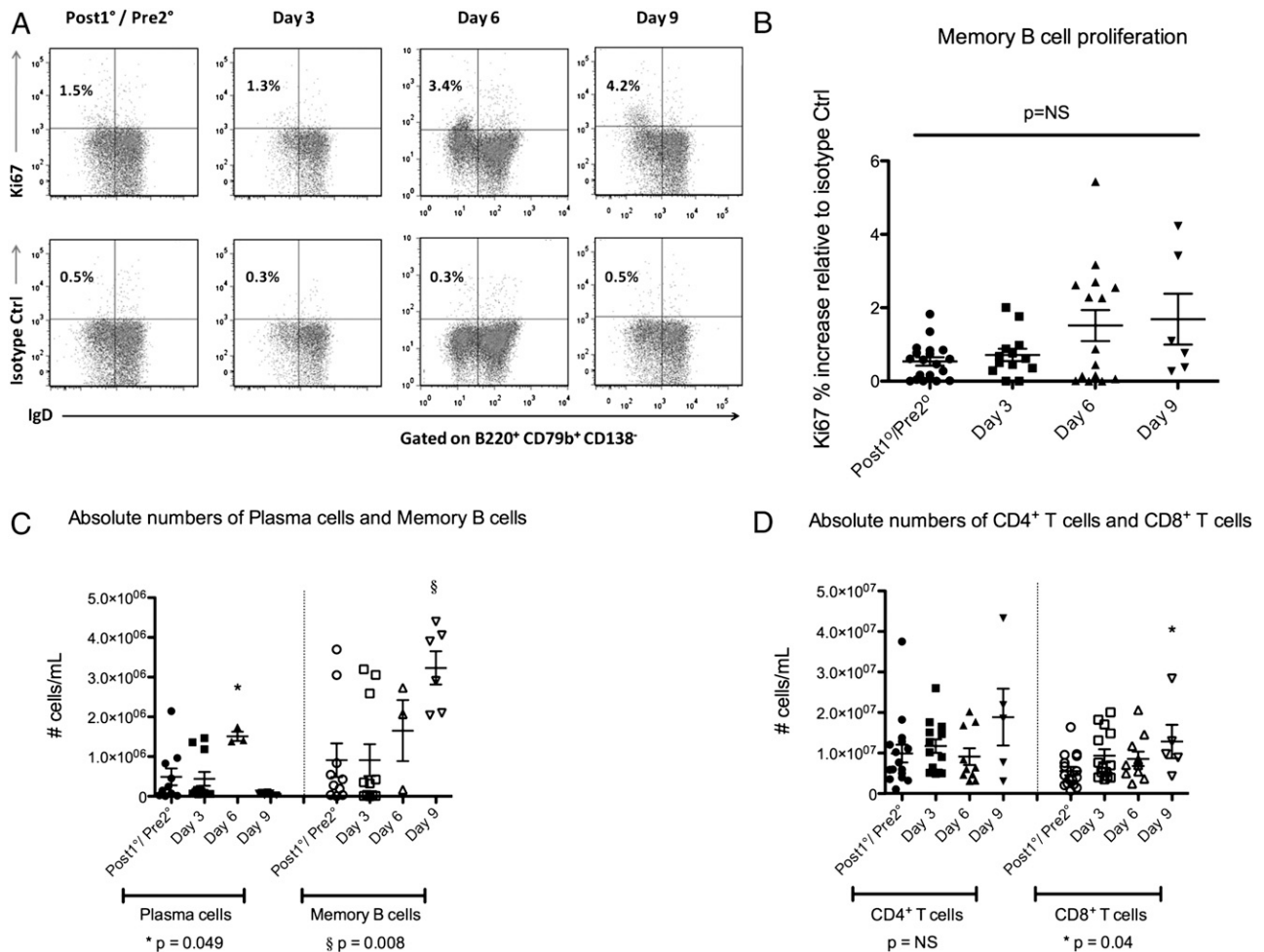


FIGURE 2. Proliferation of memory B cells and PCs after a 2° heterotypic DENV infection. *A*, Flow cytometry analysis of Ki-67⁺ memory B cells. AG129 mice were infected s.c. with 10⁵ PFU DENV-1 98J and 6–8 wk p.i. were infected i.v. with 10⁷ PFU DENV-2 D2S10. A control group was infected with DENV-1 98J and mock-infected with C6/36 cell supernatant (post-1°/pre-2°). After 3, 6, and 9 d, splenocytes were harvested and stained with anti-B220 PE-Cy7, anti-CD79b FITC, anti-CD138 PerCP Cy5.5, anti-IgD Pacific Blue, and anti-Ki-67-PE or isotype control-PE. Cells were gated on B220⁺, CD79b⁺, and CD138⁻. One representative flow cytometry staining of nine independent experiments is shown. *B*, Percentage of Ki-67⁺ memory B cells. The percentage of cells that were PE positive using the isotype control Ab was subtracted from the percentage of Ki-67-positive cells for each sample to obtain the percentage of increase relative to isotype control. Data were pooled from nine independent repeated experiments with a total of $n = 19$ mice for post-1°/pre-2°, $n = 13$ mice for day 3, $n = 15$ mice for day 6, and $n = 6$ mice for day 9. Statistical analysis was performed using the Mann–Whitney U test to compare each time point with the post-1°/pre-2° samples, and p value is shown in the graph. No significant difference was found among the groups. *C*, Absolute number of plasma cells and memory B cells. Splenocytes were treated as in *A*. Absolute numbers of plasma cells (B220^{low/-}, CD79b⁺, IgD⁻, CD138⁺) and memory B cells (B220⁺, CD79b⁺, IgD⁻, CD138⁻) in the spleen before and after 2° infection are shown. Data were pooled from six independent repeated experiments with a total of $n = 10$ mice for post-1°/pre-2°, $n = 11$ mice for day 3, $n = 3$ mice for day 6, and $n = 6$ mice for day 9. Statistical analysis was performed using the Mann–Whitney U test to compare each time point with the post-1°/pre-2° samples, and p values are shown below the graph. A statistically significant difference was found in the absolute numbers of PCs at day 6 and in the absolute number of memory B cells at day 9 p.i. when compared with the post-1°/pre-2° samples. *D*, Absolute numbers of CD4⁺ and CD8⁺ T cells. Splenocytes were treated as in *A*. Absolute numbers of CD4⁺ T cells (CD3⁺, CD4⁺, CD8⁻) and CD8⁺ T cells (CD3⁺, CD4⁻, CD8⁺) in the spleen before and after 2° infection are shown. Data were pooled from nine independent repeated experiments with a total of $n = 16$ mice for post-1°/pre-2°, $n = 14$ mice for day 3, $n = 10$ mice for day 6, and $n = 5$ mice for day 9. Statistical analysis was performed using the Mann–Whitney U test to compare each time point with the post-1°/pre-2° samples, and p values are shown beneath the graph. A statistically significant difference was observed in the absolute numbers of CD8⁺ T cells at day 9 p.i. when compared with the post-1°/pre-2° samples.

PCs, and the amount of these Abs was below the assay's limit of detection. We also could not detect any Abs in supernatants from splenocytes collected up to 9 d after a 1° DENV-2 infection, indicating that there was no Ab secreted by stimulated naive B cells that could be detected in this assay. Thus, the Abs detected in the supernatants reflect the Abs produced by memory B cells. As shown in Fig. 4F, the avidity of the splenocyte supernatant cultures increased only against DENV-1 and not DENV-2, suggesting that cross-reactive memory B cells, generated during the 1° DENV-1 infection, are the ones that proliferate and contribute to

increased avidity of the serum after a 2° heterotypic infection. Overall, these results indicate that Abs secreted by cross-reactive memory B cells are responsible for the increase in serum avidity observed during the acute phase of a 2° heterotypic infection.

Cross-reactive memory B cells generated after primary infection increase in number during a secondary heterotypic DENV infection

To quantify the number of DENV-specific B cells, total splenocytes were analyzed by ELISPOT ex vivo or after in vitro stimulation to

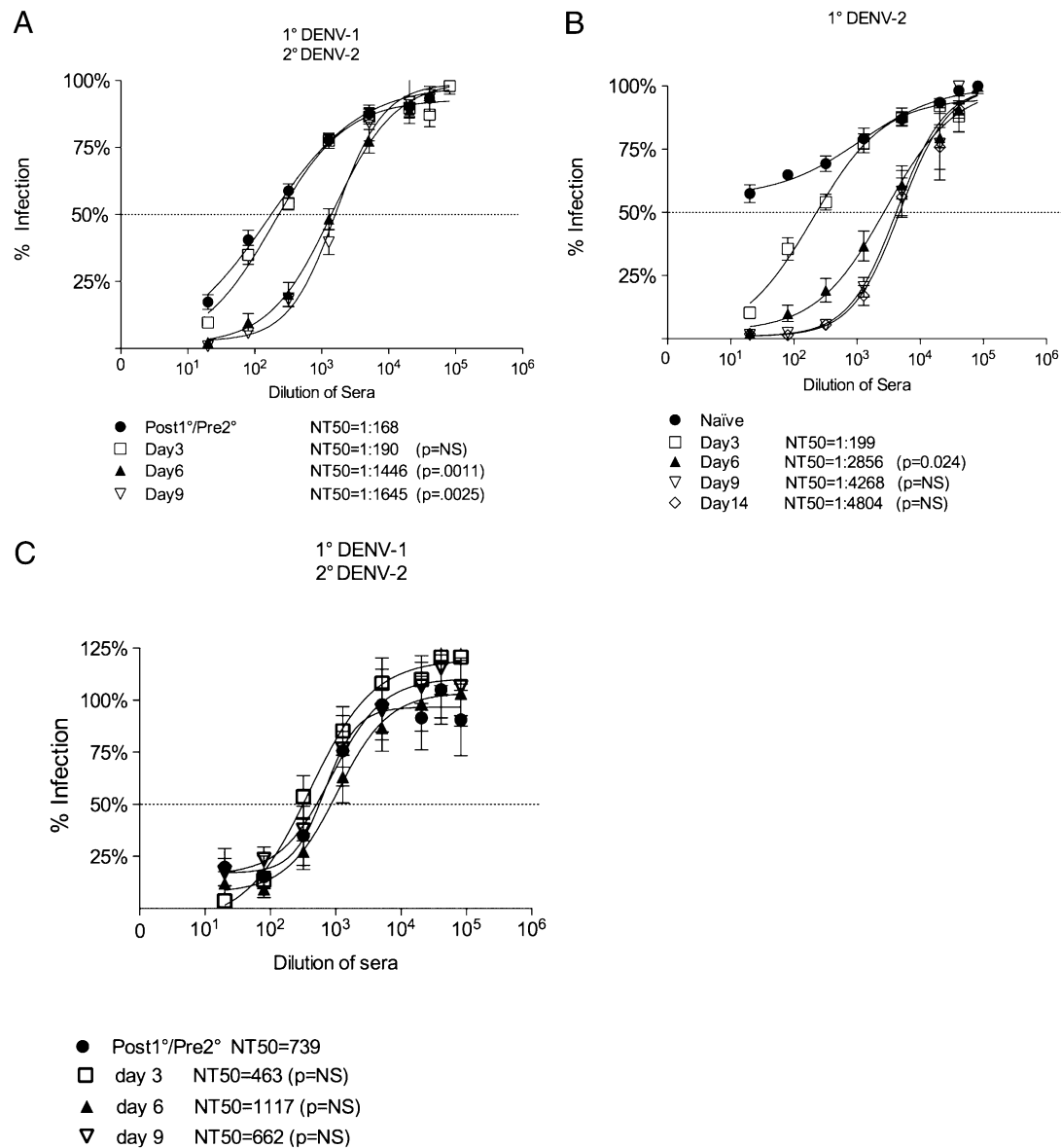


FIGURE 3. DENV-specific neutralization capacity of serum after 1° and 2° heterotypic DENV infection. **A**, Neutralizing Ab titers after 2° DENV infection. AG129 mice were infected s.c. with 10^5 PFU DENV-1 98J and 6–8 wk p.i. were infected i.v. with 10^7 PFU DENV-2 D2S10. A control group was infected with DENV-1 98J and mock-infected for the second infection (post-1°/pre-2°). After 3, 6, or 9 d, serum was collected and tested in a U937-DC-SIGN flow cytometry-based neutralization assay against DENV-2 D2S10. The dashed line corresponds to 50% neutralization, and the NT_{50} are shown in the legend. For each time point, the neutralization data pooled from four to six mice were pooled from two independent repeated experiments. Statistical analysis was performed using the Mann–Whitney U test to compare the NT_{50} of each time point with the NT_{50} of the post-1°/pre-2° serum. A statistically significant difference in NT_{50} titer was found at days 6 and 9 post-2° infection when compared with the NT_{50} of the post-1°/pre-2° serum; p values are shown in the figure. **B**, Neutralizing Ab titers after 1° DENV infection. AG129 mice were infected i.v. with 10^5 PFU DENV-2 D2S10. A control group of mice was mock-infected with C6/36 cell supernatant (Naive). After 3 and 6 d, serum was collected from DENV-2 D2S10-infected and naive mice and tested in a U937-DC-SIGN flow cytometry-based neutralization assay against DENV-2 D2S10. The dashed line corresponds to 50% neutralization, and the NT_{50} are shown in the legend. For each time point, data were pooled from three to six mice from two independent repeated experiments. Statistical analysis was performed using the Mann–Whitney U test. Each time point (days 6, 9, and 14) was compared with the day 3 serum. A statistically significant difference in NT_{50} was found at day 6 p.i. when compared with the NT_{50} of day 3 serum; p values are shown in the figure. **C**, DENV-1–specific neutralization capacity of serum after 2° heterotypic DENV infection. Neutralizing Ab titers after 2° DENV infection are shown. AG129 mice were infected s.c. with 10^5 PFU DENV-1 98J and 6–8 wk p.i. were infected i.v. with 10^7 PFU DENV-2 D2S10. A control group was infected with DENV-1 98J and mock-infected for the second infection (post-1°/pre-2°). After 3, 6, or 9 d, serum was collected and tested in a U937-DC-SIGN flow cytometry-based neutralization assay against DENV-1 98J. The dashed line corresponds to 50% neutralization, and the NT_{50} are shown in the figure. For each time point, data are pooled from five to six mice from two independent experiments. Statistical analysis was performed using the Mann–Whitney U test to compare the NT_{50} of each time point with the NT_{50} of the post-1°/pre-2° serum. No significant difference was found among the different time points.

generate ASCs. Representative pictures of the B cell ELISPOT are shown in Supplemental Fig. 1B. After in vitro stimulation, the ASCs reflect the DENV-specific memory B cells present at the time of collection, whereas the ex vivo ELISPOT reflects the DENV-specific PC population, which should be actively secreting

Ab at the time of collection. After a 2° heterotypic infection with DENV-2 D2S10 following a DENV-1 98J 1° infection, DENV-1– and DENV-2–specific memory B cells increased by ELISPOT between days 3 and 6 post-2° infection (Fig. 5A, 5B). This increase in the number of DENV-specific memory B cells correlates with

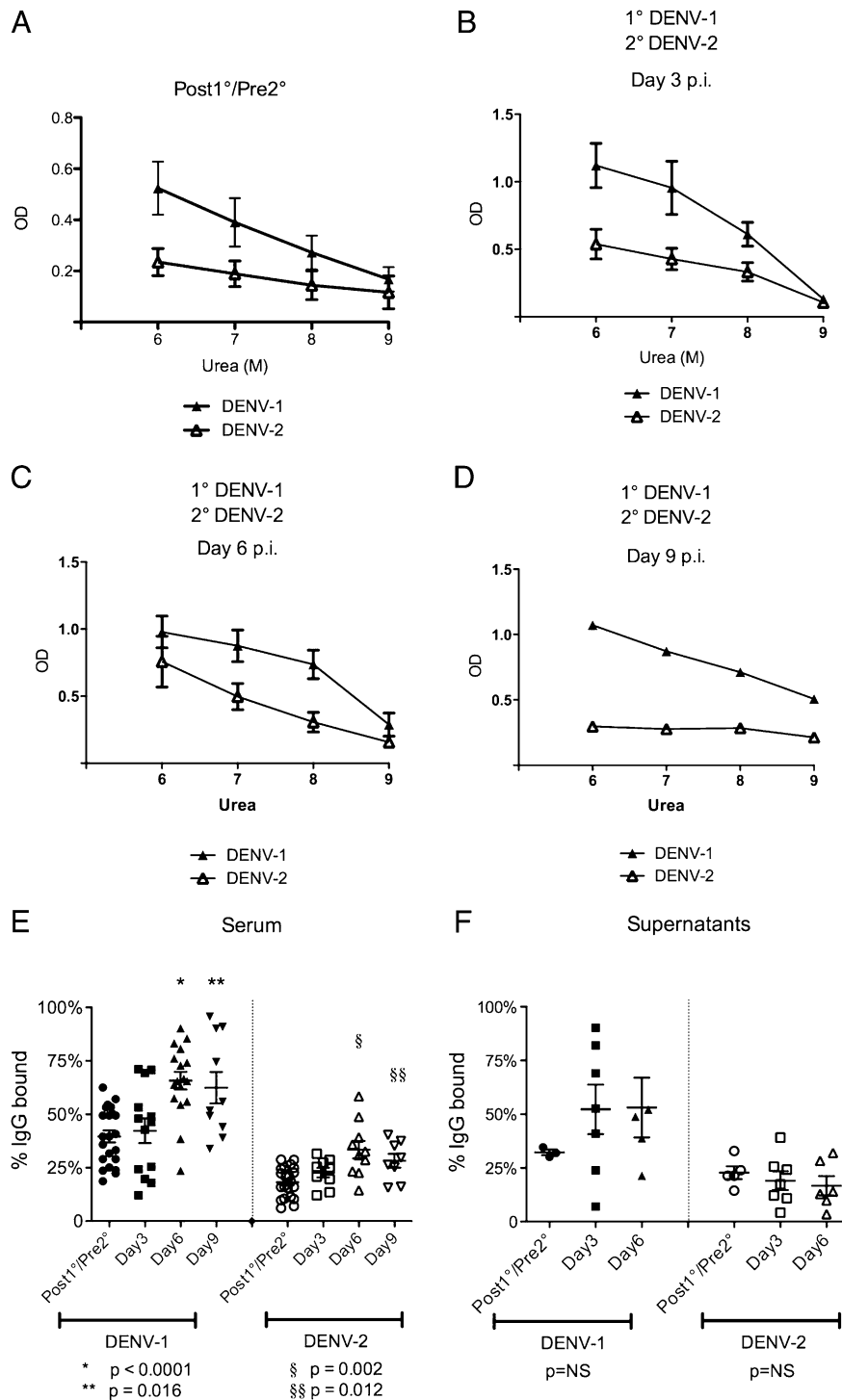


FIGURE 4. DENV-specific avidity of serum and supernatant of polyclonally stimulated splenocytes after a 2° heterotypic DENV infection. **A**, DENV-specific avidity of serum measured by ELISA using different concentrations of urea. AG129 mice were infected s.c. with 10^5 PFU DENV-1 98J and 6–8 wk later were mock-infected with C6/36 cell supernatant (post-1°/pre-2°). Serum was harvested on days 6 and 9 p.i. and tested in a urea-based ELISA assay using 6–9 M urea and recombinant E protein from DENV-1 or DENV-2 as Ag to measure avidity against DENV-1 and DENV-2, respectively. Data are pooled from three to five mice from two independent experiments. **B**, DENV-specific avidity of serum measured by ELISA using different concentrations of urea at day 3 p.i. AG129 mice were infected s.c. with 10^5 PFU DENV-1 98J and 6–8 wk later were infected i.v. with 10^7 PFU DENV-2 D2S10. Serum was harvested on day 3 p.i. and tested in a urea-based ELISA assay using 6–9 M urea and recombinant E protein from DENV-1 or DENV-2 as Ag to measure avidity against DENV-1 and DENV-2, respectively. Data are pooled from six mice from two independent experiments. **C**, DENV-specific avidity of serum measured by ELISA using different concentrations of urea at day 6 p.i. Mice were infected as in **B**. Serum was harvested at day 6 p.i. and processed as in **B**. Data are pooled from 12 mice from four independent experiments. **D**, DENV-specific avidity of serum measured by ELISA using different concentrations of urea at day 9 p.i. Mice were infected as in **B**. Serum was harvested at day 9 p.i. and processed as in **B**. Data are pooled from 12 mice from four independent experiments. **E**, DENV-specific serum avidity measured by ELISA using 7 M urea. AG129 mice were infected s.c. with 10^5 PFU DENV-1 98J and 6–8 wk p.i. were infected i.v. with 10^7 PFU DENV-2 D2S10. A control group was infected with DENV-1 98J and mock-infected with C6/36 cell supernatant (post-1°/pre-2°). Serum was harvested on days 3, 6, and 9 p.i. and tested in a urea-based ELISA assay using 7 M urea and recombinant E protein from DENV-1 or DENV-2 as Ag to measure avidity against DENV-1 and DENV-2, respectively. Data are pooled from 10–22 mice for each group from (*Figure legend continues*)

the increased proliferation and increased absolute cell numbers of the memory B cell compartment detected by flow cytometry at day 6 post-2° infection (Fig. 2A–C). Of note, DENV-1-specific memory B cells were present in greater numbers than DENV-2-specific memory B cells at day 6 post-2° infection (81.8 ± 26.8 DENV-1-specific ASC/10⁶ splenocytes versus 23.3 ± 7.6 DENV-2-specific ASC/10⁶ splenocytes, $p = 0.003$).

After a 1° infection with a sublethal dose of 10⁵ PFU DENV-2 D2S10, only 14.5 ± 4.6 DENV-2-specific ASC/10⁶ splenocytes and 37.2 ± 10.0 DENV-2-specific ASC/10⁶ splenocytes were detected at days 9 and 14 p.i., respectively (Fig. 5B). Thus, the immune response takes 14 d to generate the same number of DENV-2-specific ASCs from naive B cells after a 1° infection with DENV-2 D2S10 (37.2 ASC/10⁶ splenocytes) as detected by day 9 post-2° infection (32.7 ASC/10⁶ splenocytes), suggesting that the increase in DENV-2-reactive memory B cells during a 2° heterotypic infection is attributable to cross-reactive memory B cells induced by the first DENV-1 infection rather than naive B cells responding to the second DENV-2 infection.

After a 2° heterotypic infection with DENV-2 D2S10 following a DENV-1 98J 1° infection, DENV-1-specific PCs, as measured by ex vivo ELISPOT, peaked at day 6 post-2° infection in the spleen (149.3 ± 82.7 ASC/10⁶ splenocytes; Fig. 5D), whereas the DENV-2-specific PCs peaked at day 9 post-2° infection with lower numbers (26.3 ± 9.0 ASC/10⁶ splenocytes; Fig. 5C), suggesting that cross-reactive PCs generated from cross-reactive memory B cells or from cross-reactive semi-long-lived PCs are the main cells implicated in the humoral response after a 2° heterotypic infection as compared with homotypic PCs generated by naive B cells responding to the second infection.

After a 1° DENV-2 infection, a large number of homotypic and cross-reactive PCs are detected in the spleen (Fig. 5C, 5D). Thus, naive B cells can respond within 6 d of a 1° DENV infection by differentiating into PCs producing Abs against DENV-2 and cross-reactive Abs against DENV-1. Of interest, the peak of DENV-2-specific PCs was detected at day 6 post-1° DENV-2 infection, whereas it was detected later, at day 9, post-2° DENV-2 infection (Fig. 5D). This suggests that DENV Ag may be more readily captured by DENV-1-specific memory B cells or by anti-DENV-1-preformed Abs, thereby preventing binding and activation of naive B cells during a 2° infection. This would be expected, as memory B cells display a higher-avidity BCR than naive B cells, and suggests that DENV-2-specific PCs detected after a 2° infection arise primarily from the DENV-1-specific memory B cell pool rather than from newly stimulated DENV-2-specific naive B cells.

B cells contribute to protection against a 2° lethal heterotypic DENV infection, but are neither necessary nor sufficient for protection against lethality

AG129-immune DENV-1 mice were depleted of B cells using anti-CD20 mAb. Of note, CD20 is not expressed by PCs. Efficacy of depletion was tested prior to 2° infection in whole blood cells

isolated from an eye bleed. Less than 1% of the PBMCs were found to be CD19⁺ B220⁺ B cells in B cell-depleted mice (data not shown). At day 8 postdepletion, mice were infected a second time with a lethal dose of 10⁷ PFU DENV-2 D2S10. All the mice survived (Fig. 6A) and did not show any signs of morbidity (Fig. 6B), indicating that B cells are dispensable for protection against a lethal 2° heterotypic DENV infection. Of note, whereas B cells were completely depleted as observed by flow cytometry, and although the number of DENV-specific PCs and memory B cells decreased after B cell depletion, we could still detect DENV-specific PCs and memory B cells 6 d after a 2° heterotypic DENV infection (Supplemental Fig. 2A, Supplemental Fig. 2B), suggesting that the B cell depletion was not absolute, and, even though <1% of the PBMCs were CD19⁺ B220⁺, there were nondepleted B cells in the tissues.

B cells isolated from AG129 or WT129 DENV-1-immune mice were adoptively transferred into AG129 naive mice that were infected 24 h later with a lethal dose of DENV-2 D2S10. We isolated B cells from WT129 in addition to AG129 mice because the absence of IFN- α/β receptor could conceivably affect the recall response of memory B cells (42). The purity of the isolated B cells varied between 95 and 98% (data not shown). Although not significant, a delay in mortality was observed when comparing mice receiving B cells isolated from DENV-1-immune mice with mice receiving B cells isolated from naive mice (median survival 6.5 d [DENV-1 immune] versus 4.5 d [naive] for B cells isolated from WT129 mice and 7 d [DENV-1 immune] versus 4.5 d [naive] for B cells isolated from AG129 mice) (Fig. 7A). Of note, although mortality was delayed, mice died of vascular leak syndrome and not paralysis. Thus, cross-reactive immune B cells contribute to protection, but were neither sufficient nor necessary for full protection against a 2° lethal heterotypic infection.

T cells contribute to protection against a 2° lethal heterotypic DENV infection, but are neither necessary nor sufficient for protection against lethality

AG129 DENV-1-immune mice were depleted of CD4⁺ and CD8⁺ T cells using mAbs prior to a 2° DENV-2 D2S10 lethal infection. Efficacy of depletion was tested by flow cytometry of splenocytes isolated at day 10 p.i. after observing the mice for morbidity and mortality. No CD4⁺ or CD8⁺ T cells were found in the spleen at day 10 p.i. (data not shown). Six of seven T cell-depleted mice (85%) survived the lethal challenge (Fig. 6A) and displayed mild signs of morbidity, including lethargy, some fur ruffling, but no hunched posture between days 3 and 6 post-2° infection (Fig. 6B). In contrast, T cell-nondepleted DENV-1-immune mice did not show any signs of disease with lethal DENV-2 challenge. Overall, there was no significant difference in survival between T cell-depleted and T cell-nondepleted (PBS or isotype control) DENV-1-immune mice ($p = 0.35$). Thus, T cells contribute to protection after a 2° heterotypic DENV infection, but are not essential for protection against lethality.

nine repeated independent experiments. Statistical analysis was performed using the Mann–Whitney *U* test to compare the percentage of IgG bound after urea washes at each time point with the percentage of IgG bound after urea washes of the post-1°/pre-2° serum. A statistically significant difference in percentage of IgG bound in serum was found at days 6 and 9 post-2° infection when compared with the percentage of IgG bound in the post-1°/pre-2° serum for both DENV-1 and DENV-2 Ag; *p* values are shown below the graphs. *F*, DENV-specific avidity in supernatants from polyclonally stimulated splenocytes measured by ELISA using 7 M urea. Mice were infected as in *E*. Spleens were harvested on days 3 and 6 p.i. Splenocytes were polyclonally stimulated with PWM extract, CpG oligonucleotide, *S. aureus* Cowan, and LPS, and supernatants were collected 5–6 d poststimulation. Supernatants were tested in a urea-based ELISA using 7 M urea and recombinant E protein from DENV-1 or DENV-2 as Ag to measure avidity against DENV-1 and DENV-2, respectively. Data are pooled from five to seven mice for each group from three repeated independent experiments. Statistical analysis was performed using the Mann–Whitney *U* test to compare the percentage of IgG bound after urea washes at each time point with the percentage of IgG bound after urea washes of the post-1°/pre-2° supernatants. No significant difference was found for either DENV-1 or DENV-2 Ag.

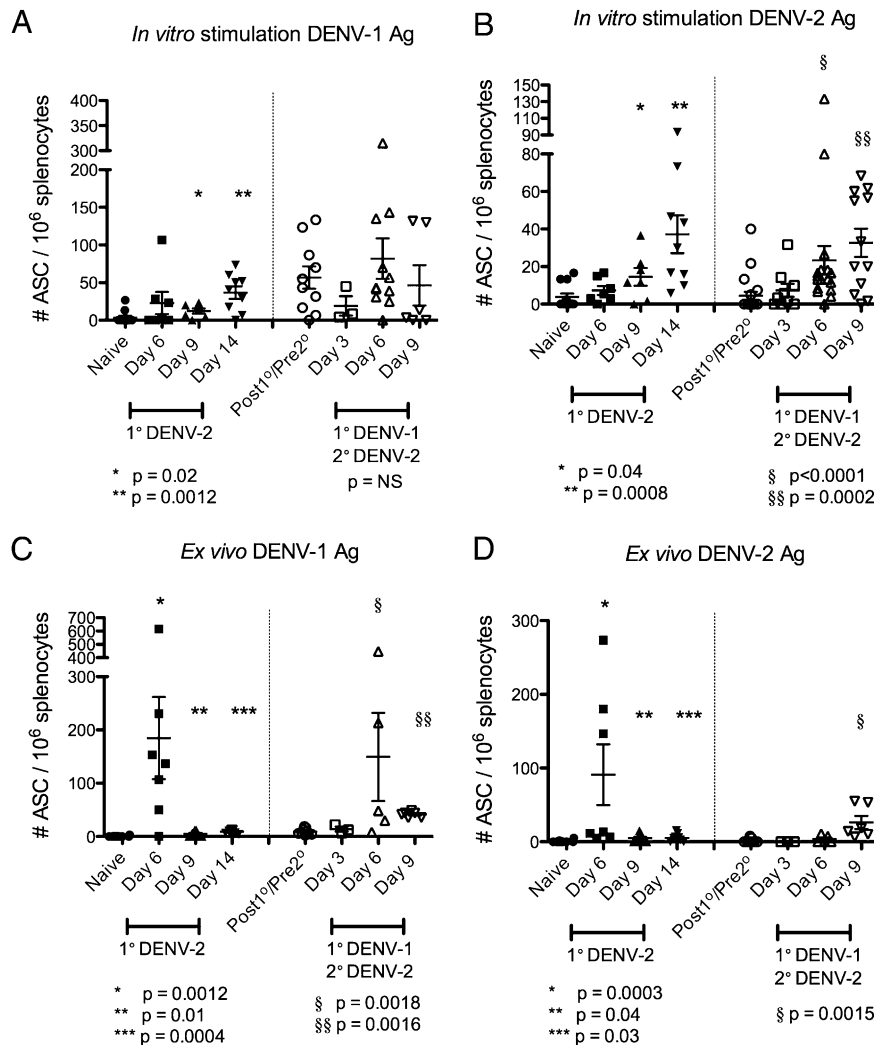


FIGURE 5. Number of DENV-specific memory B cells and PCs after 1° and 2° heterotypic DENV infection. **A**, DENV-1-specific memory B cells detected by ELISPOT. AG129 mice were infected either with 10⁵ PFU DENV-2 D2S10 i.v. (1° infection) or with 10⁵ PFU DENV-1 98J s.c. (1° infection), and then 6–8 wk p.i., infected i.v. with 10⁷ PFU DENV-2 D2S10 (2° infection). A control group was mock-infected (Naive or Post-1°/Pre-2°). After 3, 6, 9, and 14 d, splenocytes were harvested and polyclonally stimulated *in vitro* for 6 d to obtain ASCs. ASCs against DENV-1 were tested by ELISPOT using cellular Ag to detect DENV-1-specific memory B cells. The number of spots from control wells coated with mock Ag was subtracted from the number of spots counted in DENV-coated wells. Statistical analysis was performed using the Mann–Whitney *U* test to compare the number of DENV-1-specific memory B cells at each time point with the number of DENV-1-specific memory B cells from naive or post-1°/pre-2° mock-infected mice. A statistically significant difference was found at days 9 and 14 post-1° infection; *p* values are shown below each graph. **B**, DENV-2-specific memory B cells detected by ELISPOT. Cells were prepared as in **A** and were tested by ELISPOT using DENV-2 cellular Ag to detect DENV-2-specific memory B cells. Statistical analysis was performed using the Mann–Whitney *U* test to compare the number of DENV-2-specific memory B cells at each time point with the number of DENV-2-specific memory B cells from naive or post-1°/pre-2° mock-infected mice. A statistically significant difference was found at days 9 and 14 post-1° infection and at days 6 and 9 post-2° infection; *p* values are shown below each graph. **C**, DENV-1-specific PCs detected by ELISPOT. Cells were prepared as in **A** and tested *ex vivo* by ELISPOT using DENV-1 cellular Ag to detect DENV-1-specific PCs. Statistical analysis was performed using the Mann–Whitney *U* test to compare the number of DENV-1-specific PCs at each time point with the number of DENV-1-specific PCs from naive or post-1°/pre-2° mock-infected mice. A statistically significant difference was found at days 6, 9, and 14 post-1° infection and at days 6 and 9 post-2° infection; *p* values are shown below each graph. **D**, DENV-2-specific PCs detected by ELISPOT. Cells were prepared as in **A** and were tested by ELISPOT *ex vivo* using DENV-2 cellular Ag to detect DENV-2-specific PCs. Statistical analysis was performed using the Mann–Whitney *U* test to compare the number of DENV-2-specific PCs at each time point with the number of DENV-2-specific PCs from naive or post-1°/pre-2° mock-infected mice. A statistically significant difference was found at days 6, 9, and 14 post-1° infection and at day 9 post-2° infection; *p* values are shown below each graph.

CD4⁺, CD8⁺, or a mixture of CD4⁺ and CD8⁺ T cells isolated from A129 DENV-1-immune mice was adoptively transferred into AG129 naive mice, which were infected 24 h later with a lethal dose of DENV-2 D2S10. We isolated T cells from A129 mice instead of AG129 mice, as the absence of IFN- γ receptor in AG129 mice could conceivably affect the recall response of memory T cells (43). The purity of the isolated T cells varied between 76.5 and 90.5% (data not shown). No significant difference in survival was observed in mice receiving different subsets

of isolated T cells when compared with naive AG129 mice injected with PBS (Fig. 7B); however, delayed mortality was observed after CD4⁺ T cell or a mixture of CD4⁺ and CD8⁺ T cell transfers (median survival 9.0 d [DENV-1 immune] versus 4.9 d [naive] for CD4⁺ T cell and 9.5 d [DENV-1 immune] versus 4.9 d [naive] for CD4⁺ and CD8⁺ T cells). In contrast, transfer of CD8⁺ T cells alone did not delay mortality when compared with naive mice receiving PBS (median survival 5 d [DENV-1 immune] versus 4.9 d [naive]). Thus, immune cross-reactive CD4⁺ T cells

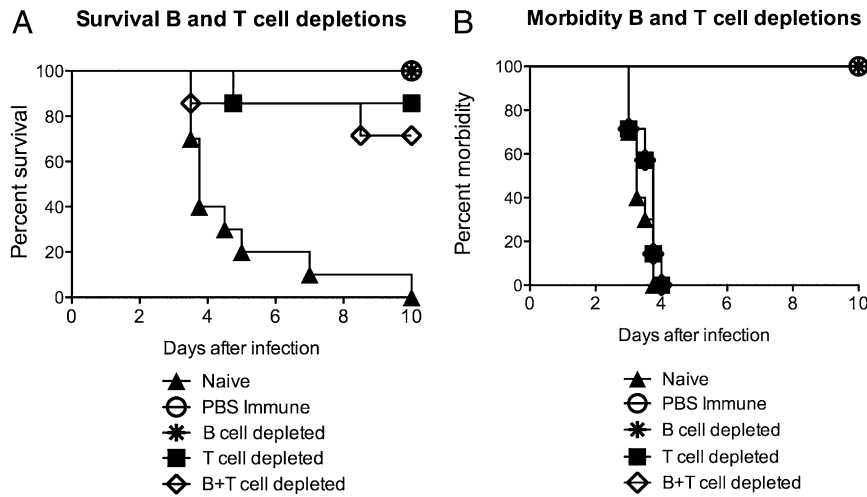


FIGURE 6. Mortality and morbidity after B and T cell depletions during 2° heterotypic DENV infection. *A*, Survival after B and T cell depletions. AG129 mice were infected s.c. with 10⁵ DENV-1 98J, and then 6–8 wk p.i. were infected i.v. with 10⁷ PFU DENV-2 D2S10. T cell-depleted mice (*n* = 7) were treated with anti-CD4 and anti-CD8 mAb, as described in *Materials and Methods*. B cell-depleted mice (*n* = 4) were treated with anti-CD20 mAb, as described in *Materials and Methods*. Four mice were depleted of both B and T cells. DENV-1-immune control mice were injected with PBS (*n* = 7) or isotype control (*n* = 3). Naive mice (*n* = 4) were infected i.v. with 10⁷ PFU DENV-2 D2S10. Data were pooled from two independent experiments. Statistical analysis was performed using the Wilcoxon rank sum test to compare the different experimental groups with the naive group. No significant differences were found. *B*, Morbidity after B and T cell depletions. The same mice as in *A* were scored for morbidity. An event was counted as soon as the mice were recorded as sick (score = 2), as described in *Materials and Methods*. Statistical analysis was performed using the Wilcoxon rank sum test to compare the different experimental groups with the naive group. No significant differences were found.

can delay mortality against a heterotypic DENV infection, but are not sufficient to protect AG129 naive mice against a lethal heterotypic infection.

Innate cellular immune response contributes to the control of initial secondary DENV heterotypic infection

AG129 DENV-1-immune mice were depleted of B and T cells using anti-CD20, anti-CD4, and anti-CD8 mAbs. Efficacy of B cell depletion was tested prior to 2° infection in whole blood cells isolated via eye bleed, and efficacy of T cell depletion was tested by flow cytometry of splenocytes isolated at day 10 p.i. after

observing the mice for morbidity and mortality (data not shown). Efficacy of B cell and T cell depletions was similar to that described above. Postinfection with a lethal dose of DENV-2 D2S10, five of seven mice (71%) survived (Fig. 6*A*) and displayed only mild signs of lethargy, some fur ruffling, and no hunched posture between days 3 and 6 post-2° infection (Fig. 6*B*). In contrast, mice receiving CP treatment died at day 7.5–8.5 p.i. (21). B and T cell depletion does not affect the innate cellular immune response, whereas CP treatment does; therefore, these results suggest that innate immune cells are also implicated in the control of a 2° heterotypic infection.

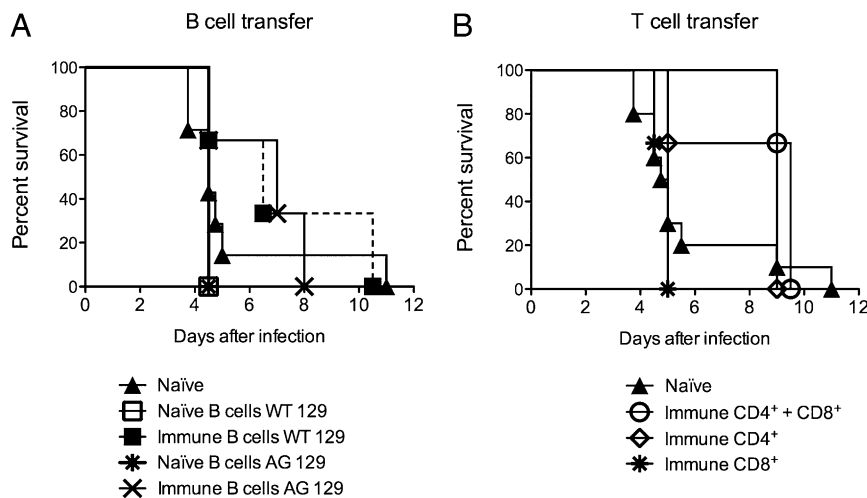


FIGURE 7. Protection against a lethal DENV-2 infection mediated by B and T cell adoptive transfers. *A*, Adoptive transfer of B cells. A total of 30 × 10⁶ B cells from AG129 or WT129 naive or DENV-1-immune mice was adoptively transferred into naive AG129 mice (3 mice/group). Control naive mice (*n* = 7) received PBS with no cells. Twenty-four hours after transfer, mice were infected i.v. with 10⁷ PFU DENV-2 D2S10. Statistical analysis was performed using the Wilcoxon rank sum test to compare the different experimental groups with the naive group. No significant differences were found. *B*, Adoptive transfer of T cells. A total of 2 × 10⁵ CD4⁺ T cells, CD8⁺ T cells, or a mixture of CD4⁺ and CD8⁺ T cells isolated from DENV-1-immune A129 mice was adoptively transferred into naive AG129 mice (3 mice/group). Control naive mice (*n* = 10) received PBS with no cells. Twenty-four hours after transfer, mice were infected i.v. with 10⁷ PFU DENV-2 D2S10. Data shown were from one experiment. Statistical analysis was performed using the Wilcoxon rank sum test to compare the different experimental groups with the naive group. No significant differences were found.

Discussion

Epidemiological studies have demonstrated the association between 2° heterotypic DENV infections and severe disease (2–5). However, most 2° infections are asymptomatic or present as classic DF, suggesting a protective role for cross-reactive Abs and the cross-reactive cellular immune response. We have previously shown that pre-existing cross-reactive Abs participate in cross-protection against a 2° heterotypic DENV infection (16, 21). In this study, we show that DENV-1-immune mice survive a lethal infection with the mouse peripherally adapted DENV-2 D2S10 strain. We found that pre-existing cross-reactive Abs, which play a role in protection, as shown by previous experiments with CP (21), were maintained by LLCs homing to the bone marrow, as shown in this study by the ELISPOT assay. During the acute phase of a 2° heterotypic DENV infection, Abs with high neutralization capacity and avidity against both DENV-1 and DENV-2 were also produced. Although highly neutralizing Abs were most probably secreted by naive B cells, highly avid Abs, as shown by our avidity assay, were most likely cross-reactive Abs produced by cross-reactive memory B cells generated after the first infection against the 1° infecting serotype. These data are in accordance with the ELISPOT data that showed a greater increase in cross-reactive memory B cells and PCs during acute 2° DENV heterotypic infection as compared with homotypic cells. Of note, no direct correlation has been shown between highly neutralizing Abs and high serum avidity to date, to our knowledge. In fact, no correlation has been found between affinity and neutralization capacities of anti-DENV mAbs (44) (K. Williams and E. Harris, unpublished data). Thus, as indicated by our neutralization and avidity assays, cross-reactive memory B cells appear to induce high-avidity Abs during a 2° heterotypic DENV infection, whereas naive B cells induce highly neutralizing Abs in response to the second DENV infection. Finally, we show that T cells and the immune innate response contribute as well to full protection against a 2° DENV heterotypic infection.

The AG129 dengue mouse model exhibits several parallels with human DENV infection and disease. The DENV-2 strain D2S10 is a mouse peripherally adapted strain that at high doses causes a lethal infection in AG129 mice by a TNF- α -dependent vascular leak syndrome, accompanied by low platelet counts, high serum cytokines, such as TNF- α and IL-10, and high levels of soluble NS1 during DENV infection (S. Balsitis and E. Harris, unpublished data) (7, 23). In this mouse model, clinical isolates from all four DENV serotypes replicate efficiently in spleen, lymph node, and bone marrow (7, 22). DENV infects relevant cells, such as macrophages, DCs, hepatocytes, and bone marrow-derived myeloid cells. Thus, the AG129 model is a relevant model to study DENV pathogenesis. Although the AG129 mice lack both IFN- α/β and IFN- γ receptors, this model reproduces both Ab-mediated protection and Ab-mediated enhancement of DENV (7, 16, 45). AG129 mice develop DENV-specific Abs that are long lasting and protective against homologous and heterologous viral challenge (16). In addition, we have shown that the IgG isotype composition of the Abs produced by AG129 is balanced (IgG1:IgG2a:IgG2b = 1:4:1) and similar to what has been reported in wild-type mice (21). Thus, the AG129 model is appropriate to study the B cell response to DENV infection in vivo. The T cell response has not been characterized extensively in these mice; however, it is known that IFN- γ is necessary for a correct recall of the memory T cell response (43). Despite this, we have previously demonstrated IFN- γ production from restimulated splenocytes collected after 2° DENV infection (16), which could be attributed to T cells. In addition, we show a certain degree of T cell functionality in our

model, as depletion of T cells prior to a 2° DENV infection induced morbidity, whereas nondepleted mice were fully protected and showed no signs of infection.

Infection with DENV confers life-long protection against infection with the same serotype, but may protect against or enhance infection with a heterologous serotype (2–5). The phenomenon of ADE (46) has been proposed, in which cross-reactive anti-DENV Abs facilitate entry of DENV into Fc γ R-bearing cells (47–49), contributing to higher viremia and disease severity. Multiple factors govern the requirements for virus neutralization, including Ab affinity/serum avidity, epitope specificity, and maturation state of the virus, as well as the interaction between specific Ab isotypes, complement, and Fc γ R (50). Thus, the same cross-reactive Abs can mediate either protection or enhancement, depending on the factors present at the time of acute 2° DENV heterotypic infection. We have previously shown that passive transfer of cross-reactive Abs is protective against a 2° DENV heterotypic infection (16) and that pre-existing cross-reactive Abs can partially mediate protection during the early phases of a 2° DENV heterotypic infection (21). We show in this study that neutralizing capacity of serum increases greatly after a 2° DENV heterotypic infection and that this increase is mediated by naive B cells.

Using a modified ELISA with urea washes, we also demonstrated that serum avidity increased after a 2° DENV heterotypic infection, but this avidity was greater against the 1° infecting serotype, suggesting that cross-reactive Abs mediate this increase in avidity. Avidity is the result of Ab affinity and valency, together with the density of viral epitopes accessible for Ab binding. Ab avidity plays an important role in the immune response against viruses and correlates with the neutralizing efficacy of Abs in vitro (51, 52). Low-avidity Abs, measured by a modified ELISA including urea washes, have been shown to provide insufficient protection against measles and respiratory syncytial virus, resulting in severe disease upon viral challenge (37, 38). Avidity ELISAs have been used to discriminate between 1° and 2° acute DENV infections in humans, as the avidity of serum to DENV increases after a 2° DENV infection (53–55). Recently, an ELISA-based competition assay measuring Ab avidity was used to characterize “original antigenic sin” in the sera of patients experiencing 1° or 2° DENV infection (56), demonstrating an increased avidity against the 1° infecting serotype. Thus, our findings of increased DENV-specific serum avidity against the 1° infecting serotype after a 2° heterotypic DENV infection is in accordance with these human studies. In addition to the studies of serum avidity, we also investigated avidity in supernatants obtained after polyclonal stimulation of splenocytes collected from mice infected with a 2° heterotypic serotype. In these supernatants, avidity increased only against the 1° infecting serotype, suggesting that these highly avid Abs, responding to the second infection, are secreted by cross-reactive memory B cells generated during the first infection. These data are in accordance with previous findings showing that after a 1° response to the haptent 4-hydroxy-3-nitrophenyl acetyl, low-affinity memory B cells, potentially cross-reactive, persist in the splenic compartment, as only 65% of the splenic memory B cells were able to produce high-affinity Abs (57). These findings correlate with our ELISPOT data showing a greater and earlier increase in DENV-1-specific memory B cells and PCs when compared with DENV-2-specific memory B cells, suggesting that cross-reactive memory B cells and PCs are active in the humoral response after a 2° heterotypic DENV infection by increasing DENV-specific avidity of the serum.

We demonstrate some amount of protection mediated by cross-reactive B cells in adoptive transfer experiments, but did not pinpoint which subcellular B cell component was implicated. The

MACS sorting method used for the B cell adoptive transfer experiment did not discriminate among B cell populations, and naive, memory B cells as well as PCs were present in the final preparation. Although long-term ASC, most probably LLPCs, are found in the bone marrow, we detected DENV-specific PCs in the spleen 6–8 wk after a 1° infection. These cells, most likely semi-long-lived PCs, were present in the cell preparation used for the adoptive transfers. We show that naive B cells alone did not mediate protection; thus, either cross-reactive memory B cells (58) or cross-reactive semi-long-lived PCs are implicated in this protection. As memory B cells proliferate between days 3 and 6 post-DENV infection, the delay in lethality observed after B cell adoptive transfer could potentially be attributed to secretion of DENV-specific Abs by the semi-long-lived PCs present in the spleen.

In the depletion studies, we used anti-CD20 mAb and did not observe any morbidity associated with B cell depletion before a 2° heterotypic DENV infection. This could indicate that the combination of pre-existing Abs and immune T cells, in association with innate immune cells, is sufficient to mediate full protection against a 2° lethal DENV infection. However, we also showed that whereas depletion was effective as measured by flow cytometry at day 8 postinjection in the blood, we could still detect DENV-specific PCs and memory B cells by ELISPOT at day 6 post-2° DENV infection. Thus, we cannot exclude that, although decreased in number, the remaining PCs and memory B cells contributed to the protection against the 2° heterotypic DENV infection observed after B cell depletion. Persistence of PCs and sustained Ab levels after B cell depletion with anti-CD20 mAb has been previously reported both in mouse and human studies (20, 40, 59).

In our model, despite the lack of IFN- γ receptors, we showed that T cells contribute to protection against a 2° heterotypic DENV infection, as shown by mAb-mediated depletion experiments. Our adoptive transfer data are consistent with previous studies. Cross-reactive CD8⁺ T cells have been implicated in the pathogenesis of severe dengue in humans (10), whereas only homotypic CD8⁺ T cells have been shown to be protective in 1° infections in mice (28). In our adoptive transfer experiments, cross-reactive DENV-1-immune CD8⁺ T cells did not mediate protection against a DENV-2 lethal infection. In contrast, adoptive transfer of CD4⁺ T cells alone or a mixture of CD4⁺ and CD8⁺ T cells mediated protection and delayed mortality. CD4⁺ T cells have been shown to be beneficial after vaccination, probably by increasing anti-DENV CD8⁺ T cell and anti-DENV Ab responses (28). Thus, adoptive transfer of immune CD4⁺ T cells alone could increase the Ab response induced by naive B cells responding to the infection, resulting in a delay in mortality. Similarly, when we transferred both CD4⁺ and CD8⁺ T cells, CD4⁺ T cells might have increased the anti-DENV CD8⁺ T cell response mediating cross-protection, even though cross-reactive anti-DENV CD8⁺ T cells transferred alone were unable to mediate protection. This suggests that, as expected, the cross-reactive anti-DENV CD8⁺ T cells required CD4⁺ T cells to be fully activated. We have recently generated a new mouse-adapted DENV-2 strain that induces morbidity and mortality in IFN- α/β receptor knockout mice (IFNAR1^{-/-}) in both the 129 and B6 backgrounds (M. Schmid, S. Orozco, and E. Harris, unpublished data). This new viral strain will allow additional T cell studies and permit testing our hypothesis about T cell cross-protection in the IFNAR1^{-/-} model.

We have previously shown that the cellular immune response is essential for full protection against a 2° heterotypic DENV infection by treating mice with CP (21). In this report, we find that after B and T cell depletion, although mice showed signs of morbidity, most of the mice treated with the depleting mAbs

survived, in contrast to mice treated with CP. In addition to abrogation of the T and B cell response, CP eliminates proliferating cells from the innate immune response, including NK cells and macrophages. We are currently analyzing the role of these cells using the IFNAR1^{-/-} mouse model.

In conclusion, this study focused on the B cell response and should be relevant for studies in human populations and research on dengue vaccines. Previous epidemiological studies have suggested immune cross-protection mediated by cross-reactive Abs (14, 15, 60). In this study, we show that cross-reactive Abs, induced by cross-reactive memory B cells, are important for protection against heterotypic DENV infection. To extend this work, we have recently adapted our ELISPOT assay to measure DENV-specific PCs and memory B cells from PBMCs in humans (S. Zompi, M. Montoya Cruz, and E. Harris, unpublished data). This current study suggests that effective vaccines should induce both robust and sustained LLPC and memory B cell responses, and analysis by ELISPOT could be explored as a correlate of protection in vaccine studies. In addition, we have shown that T cells are necessary for full protection against a 2° heterotypic DENV infection and that innate immune cells are also implicated in this protection, suggesting a complicated interplay of the different subsets of the cellular immune response. Thus, cross-reactive B cell and T cell subsets are implicated in providing cross-protection against a 2° heterotypic DENV infection, and the appearance of disease or severe disease is most probably associated with an imbalance among these immune cell subsets rather than a result of the deficiency of only one subset or the overstimulation of another.

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Disclosures

The authors have no financial conflicts of interest.

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