TITLE:

Neutralizing antibody titers against dengue virus correlate with protection from symptomatic infection in a longitudinal cohort

Leah C. Katzelnick[1, 2], Magelda Montoya[2], Lionel Gresh[3], Angel Balmaseda[4], Eva Harris[2]

[1]Centre for Pathogen Evolution, Department of Zoology, University of Cambridge

[2]Division of Infectious Diseases and Vaccinology, School of Public Health, University of California, Berkeley

[3]Sustainable Sciences Institute, Managua, Nicaragua

[4]Laboratorio Nacional de Virología, Centro Nacional de Diagnóstico y Referencia, Ministry of Health, Managua, Nicaragua

Correspondence:
Eva Harris
Division of Infectious Diseases and Vaccinology
School of Public Health
University of California, Berkeley
185 Li Ka Shing Center
1951 Oxford Street
Berkeley, CA 94720-3370
Tel. (510) 642-4845
FAX (510) 642-6350
Email eharris@berkeley.edu

ABSTRACT

The four dengue virus serotypes (DENV1-4) are mosquito-borne flaviviruses that infect ~390 million people annually; up to 100 million infections are symptomatic, and 500,000 cases progress to severe disease. Exposure to a heterologous DENV serotype, the specific infecting DENV strains, and the interval of time between infections, as well as age, ethnicity, genetic polymorphisms, and co-morbidities of the host, are all risk factors for severe dengue. In contrast, neutralizing antibodies (NAbs) are thought to provide long-lived protection against symptomatic infection and severe dengue. The objective of dengue vaccines is to provide balanced protection against all DENV serotypes simultaneously. However, the association between homotypic and heterotypic NAb titers and protection against symptomatic infection remains poorly understood. Here, we demonstrate that the titer of pre-infection cross-reactive NAbs correlates with reduced likelihood of symptomatic secondary infection in a longitudinal pediatric dengue cohort in Nicaragua. The protective effect of NAb titers on infection outcome remained significant when controlled for age, number of years between infections, and epidemic force, as well as with relaxed or more stringent criteria for defining inapparent DENV infections. Further, individuals with higher NAb titers immediately after primary infection had delayed symptomatic infections compared to those with lower titers. However, overall NAb titers increased modestly in magnitude and remained serotype cross-reactive in the years between infections, possibly due to re-exposure. These findings establish that anti-DENV NAb titers correlate with reduced probability of symptomatic DENV infection and provide novel insights into longitudinal characteristics of antibody-mediated immunity to DENV in an endemic setting.

SIGNIFICANCE

The four dengue virus serotypes (DENV1-4) are the most prevalent arboviruses worldwide and cause outcomes ranging from inapparent infection to severe disease. Neutralizing antibodies are believed to be critical for protection and therefore for dengue vaccines, but the titers required to prevent symptomatic DENV infection have not been well-established. Here, we show that higher pre-infection neutralizing antibody titers correlate with lower probability of symptomatic infection in children in a longitudinal cohort study in Nicaragua. Further, we find evidence that levels of cross-reactive neutralizing antibodies are maintained over time, possibly due to re-exposure. These findings provide insight into the determinants of DENV infection outcome and long-term immunity in an endemic setting and are relevant for dengue vaccine development.

INTRODUCTION

Dengue virus (DENV) is a mosquito-borne flavivirus that infects up to 390 million individuals each year (1). While most infections are inapparent, ~25% of infections cause acute febrile illness, which progresses to severe disease in half a million individuals annually (2). DENV consists of four evolutionarily distinct, antigenically related DENV serotypes, DENV1-4, and neutralizing antibodies (NAbs) against the four serotypes are considered a critical component of the protective immune response (3, 4). Primary (1°) DENV infection induces a NAb response that is described as increasingly type-specific over time, providing long-term protection against the 1° infecting serotype but only transient protection against other DENV serotypes (5, 6). Cross-serotype protection against symptomatic infection is observed for up to two years after 1° infection, after which point individuals are at increased risk of symptomatic infection and severe dengue upon subsequent heterologous infection (7–10). Over time, cross-serotype-reactive antibodies are thought to decay to sub-neutralizing levels, binding but not neutralizing DENV and contributing to enhanced replication during heterologous infection by facilitating virus entry into target cells expressing Fc receptors (11). However, following subsequent infection with a different serotype, the NAb response becomes broadly neutralizing, and is thought to reduce incidence of severe disease (12).

There has been limited success in establishing the relationship between the level of pre-infection NAb titers to DENV and risk of disease upon subsequent DENV infection in endemic settings. In recent vaccine trials, symptomatic disease was observed in individuals with relatively high NAb titers, raising concerns that the current immunologic assays do not measure the NAbs critical for protection (13). In studies of infants, who receive IgG antibodies by trans-placental transfer from DENV-immune mothers, infants with higher NAb titers at birth generally, although not always, experienced symptomatic disease later than those with lower titers (14–16). Recent studies in children and adults have made important advances in demonstrating an association between the quantity of cross-reactive pre-infection NAb titers and reduced risk of symptomatic secondary (2°) infection, defined as $\geq$2 infections, but have not been conclusive: the association did not hold for all DENV serotypes (15, 17), exposure could not be proven for DENV-negative individuals (18), or the magnitude of pre-infection NAb titers was not directly studied (12, 19). Thus, there is an urgent need to definitively establish whether NAb titers correlate with protection in endemic settings. Here, we estimated the relationship between pre-infection NAb titers and probability of symptomatic infection and characterized determinants of long-term

protection in children with multiple DENV infections in a pediatric dengue cohort study in Nicaragua.

RESULTS

Selection of the repeat infection sample set. The Nicaraguan Pediatric Dengue Cohort Study (PDCS, 2004-present) is a community-based study with "enhanced" passive surveillance with an average active cohort of ~3,500 children (7,547 to date) aged 2-14 years in Managua. Healthy annual blood samples are collected from all participants each year (in July/August prior to 2011 and March/April since 2011). Symptomatic infections are confirmed in children who present to the study Health Center with suspected dengue or undifferentiated febrile illnesses by RT-PCR and/or virus isolation in acute samples, and/or serological assays in paired acute and convalescent samples (20). Inapparent infections are identified using Inhibition ELISA (IE) on paired healthy annual samples processed side-by-side each year, defined as seroconversion (1° infections) or a ≥4-fold increase in anti-DENV antibody titer (2° infections) (21). By IE, we capture ~80% of symptomatic infections, as reported previously (21). In total, 1,114 children were DENV-naïve at enrollment and experienced ≥1 infection. From this group, we assembled the "repeat infection sample set" by randomly selecting 62/224 with 2 infections and 32/54 with 3 infections as identified by either IE or symptomatic infection; 18/834 with 1 infection were also selected for comparison (n=112 total) (9). Children in the sample set were an average of 6.19 years old at the time of their first DENV infection, and 45% were female (see SI Appendix, Table S1).

To reconstruct the immunological history of each child in the repeat infection sample set, NAb titers to the four DENV serotypes were measured in annual samples from all available years. NAb titers were measured by endpoint titration and calculated as 50% neutralization (NT_{50}) in a flow cytometry-based assay using human Raji-DC-SIGNR cells with reporter virus particles (RVPs) representing the four DENV serotypes (9, 22). Titrations were only accepted once they met stringent quality control standards (see Methods) (9).

Inapparent infections were classified using the same criteria as previously reported (9): a ≥4-fold increase in NAb titer between annual samples to a DENV serotype other than a previous infecting DENV serotype or a serotype that later caused a symptomatic infection (hereafter called the standard infection criteria). Based on analyses of reproducibility of positive control

titrations, we expect identical serum samples titrated against all four DENV types at two time-points to exhibit a ≥4-fold increase in titer for at least one of the four measured titers, and thus incorrectly be coded as an infection, in only 0.9-1.0% of comparisons (see SI Appendix, Fig. S1). Compared with inapparent infections identified by applying standard infection criteria to the NAb titers, inapparent infections identified by IE had a specificity 95% and a sensitivity of 64%. With standard infection criteria, we identified 22 symptomatic 1°, 71 inapparent 1°, 38 symptomatic 2°, and 87 inapparent 2° DENV infections. Based on NAb responses, the children were grouped into the following subsets: subset 1 entered the study DENV-naïve and had ≥2 infections (n=69); subset 2 entered the study DENV-immune and ≥1 infection (n=19); and subset 3 entered the study DENV-naïve and had only 1 infection (n=24) (see SI Appendix, Table S2).

We also identified the infecting serotype as described in Methods. The serotype identified by RT-PCR corresponded to the greatest *change* in NAb titers in 94% of symptomatic 2° infections, and 41% exhibited original antigenic sin, defined as a post-infection NAb *titer* higher to the 1° than to the 2° infecting serotype. For inapparent 2° infections, we identified the infecting serotype by considering the magnitude of the change in NAb titer as well as the epidemiology of DENV in the year of infection, as others have done (10), based on DENV infection data for the full PDCS (21). With these criteria, 76% of infections had the largest change in NAb titer to what was identified as the 2° infecting serotype, and 60% exhibited original antigenic sin.

Pre-infection neutralizing antibody titer predicts infection outcome. We first tested whether higher levels of cross-reactive NAb titers reduced the probability of symptomatic infection by estimating the relationship between pre-infection NAb titers measured in the annual sample before the 2° infection and symptomatic versus inapparent 2° DENV infections for subset 1, using single and multiple logistic regression. Pre-infection NAb titers were estimated as the median of NAb titers to the four serotypes, and covariates included age at 2° infection, number of years between 1° and 2° infection, or "epidemic force", a metric based on the annual ratio of symptomatic to inapparent (S:I) infections in the full PDCS (See Methods and SI Appendix, Fig. S2).

In all multiple logistic regression models, children with a higher median pre-infection NAb titer were significantly less likely to experience symptomatic 2° infections (Fig. 1A, Table 1; see SI Appendix, Tables S3, S4). When covariates in all models were set to the average value observed

in the sample, the probability of symptomatic infection decreased with higher levels of pre-infection median NAb titer (Fig. 1B). Based on the model "median NAb titer + epidemic force", individuals with a titer of 1:260 have a 10% probability of symptomatic infection in an average year. We tested the predictive ability of the model "median NAb titer + epidemic force" with cross-validation (see Methods). Overall, the predicted probabilities corresponded well to the observed proportions of symptomatic DENV infections for each estimated probability group, similar to model parameters estimated with all children in subset 1 (see SI Appendix, Table S5). We expanded our analyses of protection to also include subset 2 (DENV-immune upon study enrollment) and found that the protective effect of median pre-infection NAb titer remained significant (see SI Appendix, Table S6). In contrast, the pre-infection NAb titer to the 1° infecting serotype was not significantly associated with infection outcome, suggesting that the heterotypic NAbs were providing protection (Table 1; see SI Appendix, Tables S3-S4, S6).

Alternative criteria for identifying inapparent DENV infections do not change the relationship between median pre-infection NAb titer and infection outcome. We conducted sensitivity analyses to determine whether the protective effect of median pre-infection NAb titer was robust to the criteria used to identify inapparent infections. Using "relaxed" infection criteria, defined as any ≥4-fold increase in NAb titer, thus allowing for re-infection with previous infecting serotypes, those in subset 1 with a higher median pre-infection NAb titer were at reduced risk of symptomatic disease (Table 1; see SI Appendix, Tables S2-S4). The relationship remained when also analyzing subsets 2 and 3 with the "relaxed" infection criteria (see SI Appendix, Table S6). We then applied more stringent criteria for inapparent infections, which consisted of the standard criteria as well as a median NAb titer increase of >1-fold. Although this reduced the number of inapparent infections, the effect of median pre-infection NAb titer on infection outcome remained (Table 1; see SI Appendix, Tables S3-S4, S6). We concluded that potential misclassification of inapparent infections would not substantively alter the measured relationship between NAb titer and protection.

Pre-infection neutralizing antibody titer to the 2° infecting serotype predicts infection outcome. We next tested whether the pre-infection NAb titer to the 2° infecting serotype was predictive of infection outcome. We found that, controlling for epidemic force or years between 1° and 2° infections, the NAb titer to the 2° infecting serotype was also associated with reduced likelihood of symptomatic infection (Table 1; see SI Appendix, Tables S3-S4, S6, Fig. S3A-C).

The effect size for the model "NAb titer to 2° serotype + epidemic force" remained similar when relaxed and stringent infection criteria were used (Table 1; see SI Appendix, S3-S4, S6), and also performed well in cross-validation analyses (Table S5).

Epidemic force is the strongest independent predictor of symptomatic infection. We found that infection during years with a strong epidemic force, at older age, and with more time between 1° and 2° infection were all significantly associated with a higher probability of symptomatic 2° infection (Table 1). However, the logistic model with "NAb titer + epidemic force" was more parsimonious (Akaike Information Criterion, AIC=102.2) than those with either age (AIC=117.0) or years between infections (AIC=116.5), and only epidemic force remained a significant independent predictor of symptomatic disease when controlling for age and years between 1° and 2° infections in the same regression model (See Methods and SI Appendix, Table S7). DENV serotypes, genotypes, and strains differ their capacity to cause symptomatic/severe disease as well as large epidemics (23, 24). The 2009-2010 epidemic season, when DENV3 genotype III was circulating in Nicaragua (21, 25), had a strong epidemic force and thus a large independent risk of symptomatic disease (see SI Appendix, Fig. S2). When each model covariate was set to the value observed in 2009-2010, the effect of NAb titer on the probability of symptomatic infection was more striking (Fig. 1C) than that observed for average covariate values (Fig. 1B).

Primary infection outcome modifies 2° infection outcome. We also tested whether the infecting DENV serotype and clinical outcome of the 1° infection modified the probability of symptomatic 2° infection in the model, "NAb titer + epidemic force". Children with symptomatic 1° infections were less likely to have symptomatic 2° infections independent of NAb titer (median or 2° serotype), suggesting that symptomatic 1° infection may modify subsequent disease risk in a way not fully measured by NAb titers. In contrast, we did not observe a significant relationship between either the 1° serotype or the 2° serotype and symptomatic 2° infection (see SI Appendix, Table S8).

Higher titers immediately after 1° infection delay the occurrence of symptomatic infection. In studies of symptomatic dengue in infants of DENV-immune mothers, infants with later symptomatic infections have higher NAb titers at birth than those infected earlier (14–16), suggesting that infants exposed after maternal antibodies had decayed to low levels were at

greater risk. We tested this hypothesis for subset 1 to determine whether children with higher NAb titers to the 2° infecting serotype immediately after 1° infection were protected for a longer period of time against symptomatic infection. We found that every two-fold higher NAb titer to the 2° serotype, as measured immediately after 1° infection, provided a six-month delay to symptomatic infection (Fig. 2A). We hypothesized that NAb titers would decay to low levels in all individuals in the year prior to their symptomatic 2° infections. However, we did not observe a significant difference in NAb titers to the 2° infecting serotype from immediately after 1° infection to the year before 2° symptomatic infection (paired t-test, 1.23-fold increase, $p=0.21$) and children with later 2° infections, both symptomatic and inapparent, had more $\geq$4-fold increases in NAb titers between their 1° and 2° infections than those with earlier 2° infections ($p<0.001$, linear regression). Yet overall, those with 2° inapparent infections still consistently had higher pre-infection NAb titers than those with 2° symptomatic infections (Fig. 2B; see SI Appendix, Fig. S3D). These observations suggest that the increase in NAb titers may have been due to intermediate DENV exposures.

Neutralizing antibody titers increase in magnitude and breadth following 1° infection. In the absence of re-infection with DENV, post-1° infection NAb responses are expected to decay and become increasingly type-specific over time (5). We measured the magnitude and breadth of NAb titer trajectories following 1° infection and before the subsequent exposure to DENV (as identified with standard criteria) for subset 1 ($\geq$2 infections) and subset 3 (1 infection) (see Methods). In both subsets 1 and 3, we observed that the magnitude of NAb titers between 1° and subsequent infection increased modestly, but significantly (Fig. 3; see SI Appendix, Table S9). A similar phenomenon was seen for the breadth of the NAb response: those in subset 1 became slightly more cross-serotype neutralizing over time, while subset 3 had stable NAb responses. In subset 1, we also measured the trajectories of NAb titers between 2° infections defined with standard criteria. NAb titers modestly declined in magnitude, but had stable cross-serotype neutralization over time (Fig. 3; see SI Appendix, Table S9).

DISCUSSION

We have found that pre-infection NAb titers reduce the risk of symptomatic DENV infection in a longitudinal dengue cohort. We observed that a higher median pre-infection NAb titer or NAb titer to the 2° infecting serotype, but not the NAb titer to the 1° serotype, was significantly associated with reduced probability of 2° symptomatic DENV infection, indicating that cross-

reactive NAbs determine protection, as others have proposed (7, 8, 12, 17, 19). The protective effect of pre-infection NAb titers remained when the criteria for inapparent infections were relaxed or made more stringent, and the models performed well in cross-validation experiments, reinforcing the strength of this observation. We also found that higher NAb titers to the 2° infecting type immediately after 1° infection delayed symptomatic 2° infections, but that this effect could not be attributed primarily to antibody decay, as NAb titers increased modestly in magnitude and remained serotype cross-reactive in the years between 1° and 2° infection. Collectively, our findings establish that higher levels of cross-reactive pre-infection NAb titers correlate with reduced probability of symptomatic DENV infection, and that NAb titers do not become increasingly type-specific over time in endemic settings.

Our findings advance those of previous studies to establish the relationship between the quantity of NAbs and protection against DENV in an endemic setting. Previous longitudinal cohort studies in Thailand found that pre-infection NAb titers reduce the risk of severe disease (17), while studies in Sri Lanka and Peru found that the number of DENV serotypes that were detectably neutralized reduced the risk of symptomatic compared with inapparent 2° DENV infection (12, 19). A recent index-cluster study in Thailand found that individuals with symptomatic infections had lower pre-infection NAb titers than a comparable group of individuals likely, but not definitely, exposed to DENV but who did not become ill (18). However, due to study limitations, these findings collectively do not provide definitive proof of the relationship between NAb titers and protection: the protective effect was not consistently observed against all DENV serotypes (17), the DENV-negative individuals may not have been exposed to DENV (18), and the magnitude of NAb titers was not directly analyzed (12, 19). Beyond dengue cohort studies, there remains concern that NAb titers measured with non-human cell substrates are not as biologically relevant (13). Our study fills a critical gap in the literature, establishing that higher levels of pre-infection NAb titers reduce the risk of symptomatic compared with inapparent DENV infection in a longitudinal cohort of children infected repeatedly over multiple years, as measured using a statistically robust neutralization assay on a human cell substrate. Future studies that measure other functional properties of NAbs will be important for establishing the mechanism of such neutralization.

We observed that age, interval between 1° and 2° infections, and epidemic force in the year of 2° infection were independent predictors of 2° infection outcome. However, in models including

epidemic force and either age or years between 1° and 2° infections, only epidemic force remained significant, suggesting that it was the major independent predictor of infection outcome besides pre-infection NAb titer. Previous studies have found that the time between 1° and 2° infections is a strong predictor of infection outcome (7–9). However, in other studies, age and years between infections were used as an indirect measure of NAb titers (older children are more likely to have higher NAb titers, and more years between infection may correlate with antibody decay), whereas we have estimated the effect of NAb titer on infection outcome directly. Interestingly, Anderson *et al.* (7) observed an effect of time on infection outcome independent of antibody response, suggesting that perhaps an effect of epidemic force has been observed in other settings.

Epidemic force is likely a function of whether a major epidemic occurred recently, the virulence/transmissibility of the infecting serotype and strain, and the antigenic properties of the strain in relation to population immunity and the serotypes and strains that circulated previously. That there are major fluctuations in the annual S:I ratio is well established and has been observed by all longitudinal cohort studies conducted to date (26). The DENV serotypes are also observed to have intrinsic differences: DENV1 and DENV3 cause more symptomatic/severe primary infections than DENV2 and DENV4, DENV2 causes the most symptomatic/severe secondary infections, DENV3 is associated with the largest epidemics, and DENV4 infections tend to be mild or subclinical (23, 24, 27, 28). Within serotypes, there may also be differences in epidemic potential of genotypes or clades, due to either their antigenic properties in relation to population immunity or their intrinsic fitness differences (28–31). Of note, the years with the strongest epidemic force described here coincided with the re-introduction of DENV3 into Nicaragua, similar to what occurred in Iquitos, Peru, in 2001-2002, where DENV3 was introduced into a population previously only infected with DENV1 and DENV2, leading to an initial high S:I infection ratio (24). Our findings suggest that the NAb titer that distinguishes those with a high probability of symptomatic compared with inapparent infection may depend on the annual S:I ratio and thus may vary in relation to the risk of disease given DENV infection from year to year (29–31). This raises the possibility that there may not be a single NAb titer that correlates with protection from symptomatic infection, even for each DENV serotype, and that it may differ from epidemic to epidemic.

We also found that the 1° infection outcome independently predicted subsequent symptomatic DENV infection. We did not observe any significant differences in 2° infection outcome by 1° or 2° infecting serotype, but our study was not powered to test the specific relationship between serotypes. The sequence of infecting serotypes has been shown to be important in other cohorts and settings (29, 30). In future studies using higher resolution antigenic and epidemiological data, it may be possible to identify the specific antigenic relationship between the 1° and 2° infecting DENV isolates, particularly if using Nicaraguan strains, rather than prototype isolates, for the neutralization assays (32).

Our findings also provide insight into the maintenance of protective NAbs in endemic settings. We found that children with higher NAb titers immediately after 1° infection were protected for longer against symptomatic infection than those with lower titers, but this effect does not appear to be due to antibody decay, as NAb titers modestly increased between the year after 1° infection and the year before 2° infection. Our study was conducted in an endemic setting, whereas previous studies of neutralizing responses in individuals long after 1° infection were conducted in non-endemic areas (5). However, in a comparable hospital-based study following pediatric dengue cases in Nicaragua, NAb titers declined in magnitude between 2 weeks and 6 months post-infection, but increased between 6 to 18 months post-infection for a subset of individuals, suggesting subsequent DENV-exposure (33). These observations raise the possibility that many children in the Nicaraguan cohort were exposed to DENV more often than is measured using standard infection criteria.

The frequency of boosts in NAb titer observed in the repeat infection sample set suggest that some may have been caused by re-infection with the 1° DENV serotype. This is consistent with the epidemiology of DENV in Nicaragua, where one DENV type generally dominates for multiple years at a time. Currently, only heterologous DENV infections are thought to cause infection, as the dogma is that individuals have sterilizing immunity to all variants of the 1° infecting serotype (34). However, recent experimental studies in non-human primates demonstrate that inoculation with either the same or different genotypes of DENV2 one year after 1° DENV2 inoculation can cause a persistent boost in NAb titers as measured by $PRNT_{50}$, particularly if individuals had low NAb titers prior to the DENV2 challenge (35). Our findings suggest that DENV re-exposure may be important for maintaining long-term humoral immunity in endemic settings; individuals may have sufficient antibodies to quickly control infection, but

experience sufficient replication to stimulate the immune memory cell population and potentially increase the quantity of circulating NAbs. Nagao and Koelle observed in modeling studies that a decline in DENV transmission was associated with increased incidence of severe disease, and posited that frequent exposure to DENV may be required to maintain sufficient levels of NAbs for sustained protection against disease (36).

There are a few considerations for the interpretation of our findings. The repeat infection sample set is only a subset of the Nicaraguan cohort and thus the effect sizes of parameters estimated here may differ if studied in the larger cohort. Specifically, selection for the repeat infection sample set may have led to oversampling of individuals with a high rate of DENV exposure. Additionally, the 2007-2008 DENV2 epidemic, which caused a disproportionate number of severe 2° cases in the full cohort (29), is mostly absent in the repeat infection sample set because only children who were DENV-naïve at enrollment were included. More generally, our findings are bound by the specific epidemiology of dengue in Nicaragua over the time-period studied.

Our findings have potential implications for vaccine development and implementation. Vaccines that generate higher levels of NAb titers will potentially reduce the probability of symptomatic infection, but the level of NAbs required for protection against symptomatic disease may differ from year to year in endemic settings. Further, a vaccine that induces a sufficiently strong NAb response to protect against DENV infection for at least a few years may be successful in endemic areas, if indeed frequent re-exposure helps maintain high levels of NAbs. However, the same vaccine might be less effective in areas with infrequent outbreaks or in travelers if NAb titers indeed wane in non-endemic settings.

Overall, we demonstrate that high NAb titers are associated with reduced probability of symptomatic infection in DENV-endemic areas. Further, our data suggest that regular DENV re-exposure, including to homologous DENV types, may help maintain these high levels of NAb titers over time.

MATERIALS AND METHODS

Neutralization titrations. NAb titers were measured as 50% neutralization (relative to no antibody control) in a flow cytometry-based assay using human Raji-DC-SIGNR cells with reporter virus particles (RVPs) representing the four DENV serotypes: DENV1, Western Pacific

74; DENV2, S16803; DENV3, CH53489; DENV4, TVP360 (9, 22). Where possible, all annual samples for each child were titrated side-by-side. The raw antibody titration data were fitted with a two-parameter (slope and intercept) sigmoidal dose-response curve to estimate the NAb titer (NT_{50}). The RVP concentrations were tested to ensure they abided by the law of mass action. Quality control standards were implemented for the sigmoidal dose-response regression fit, including an absolute sum of squares <0.2 and the coefficient of determination (R^2) >0.9 (9).

Identifying the infecting serotype. If an individual had a ≥4-fold increase to only one serotype, that serotype was identified as the infecting serotype. However, in Nicaragua, generally one DENV serotype causes the majority of infections in any given year. Thus, if the individual also had a >1.5-fold increase in NAb titer to a serotype with higher incidence in that year (based on incidence data for the full PDCS), the higher-incidence serotype was identified as the infecting serotype. We conducted sensitivity analyses and found that the effect sizes and p-values estimated with different fold-increases (>1-fold, >2-fold, or >4-fold) were similar to those estimated with >1.5-fold increase.

Epidemic force. The epidemic force was estimated as the annual ratio of symptomatic to inapparent (S:I) infections in the full PDCS divided by the average of the S:I ratios across all years (2004-2014) to provide a relative, independent measure of risk of symptomatic infection in Managua, Nicaragua, for each year (see SI Appendix, Fig. S2). As a sensitivity analysis, we also defined epidemic force using only the S:I ratio for 1° infections in the whole PDCS and obtained similar results (see SI Appendix, Table S4).

Magnitude and breadth. Magnitude was estimated as the mean of NAb titers to all four DENV serotypes for each individual at each time point. Breadth was estimated by identifying the best and second-best neutralized DENV serotypes in the year immediately after 1° infection, and measuring the ratio of the two NAb titers for each individual at each time point.

Statistical analyses. All statistical analyses were conducted using R version 3.1.3. We used logistic regression to test the association between median pre-infection NAb titers *or* the NAb titer to the 2° serotype and 2° infection outcome, controlling for age at 2° infection, years between 1° and 2° infection, or epidemic force. Effects were estimated as the average marginal effect (AME, which allows comparison between models (37)), odds ratios (OR), or the

probability of symptomatic infection (the OR/(1+OR)). We conducted cross validation of the model "NAb titer + epidemic force" by estimating model parameters with data from a randomly selected sample of 90% of children in subset 1 and used the resulting model to predict the probability of symptomatic infection for the excluded 10%, repeating this process 10,000 times, using different random subsets. We used the AIC to compare logistic regression models with pre-infection NAb titer and age, years between 1° and 2° infections, or epidemic force. We built logistic regression models with multiple covariates to test the importance of each in relation to 2° infection outcome. We estimated the relationship between NAb titers and years until 2° infection with linear regression. Fold-changes in magnitude and breath were estimated with linear regression, controlling for each individual's initial NAb responses with individual intercept values. Separate models were run for all individuals with at least 2 years, 3 years, etc. of follow-up.

ACKNOWLEDGEMENTS

We thank Aravinda de Silva, Hannah Clapham, and Judy Fonville for helpful comments on the manuscript, and Ana Mosterin, Aubree Gordon, Derek Smith, and Steve Whitehead for useful discussions. We thank past and present members of the study team based at the Centro de Salud Sócrates Flores Vivas, the National Virology Laboratory in the Centro Nacional de Diagnóstico y Referencia, and the Sustainable Sciences Institute for their dedication and high-quality work. We are also extremely grateful to the study participants and their families. This work was supported by the FIRST (Fighting Infections through Research, Science, and Technology) grant from the Bill and Melinda Gates Foundation and the Instituto Carlos Slim de la Salud (to EH) and the National Institute of Allergy and Infectious Diseases of the National Institutes of Health (R01 AI099631 to AB and P01 AI106695 to EH). The Nicaraguan Pediatric Dengue Cohort Study was also supported by the Pediatric Dengue Vaccine Initiative grant VE-1 (to EH). LCK was supported by Gates Cambridge and the NIH Oxford-Cambridge Scholars Program.

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Table 1. Pre-infection NAb titers and likelihood of symptomatic 2° infection, controlling for epidemic force, age, or years between 1° and 2° infection.

	Multiple logistic regression models (subset 1)											
	NAb titer + epidemic force				NAb titer + age				NAb titer + yrs. 1° to 2°			
Variable (Infection criteria)	*OR*[1]	*p*	*AME*[2]	*p*	*OR*	*p*	*AME*	*p*	*OR*	*p*	*AME*	*p*
Median NAb titer (std.[3])	0.67	0.01	-0.07	0.04	0.74	0.03	-0.06	0.05	0.64	0.01	-0.09	0.02
Covariate[4] (std.)	4.40	<0.001	0.24	<0.01	1.21	0.03	0.04	0.05	1.41	0.02	0.07	0.04
Median NAb titer (rel.)	0.66	0.01	-0.07	0.03	0.75	0.03	-0.05	0.05	0.66	0.01	-0.08	0.02
Median NAb titer (str.)	0.73	0.07	-0.06	0.10	0.83	0.23	-0.04	0.25	0.66	0.02	-0.09	0.05
Nab titer 1° serotype (std.)	1.01	0.90	0.00	0.90	0.97	0.78	-0.01	0.78	0.93	0.48	-0.02	0.49
NAb titer 2° serotype (std.)	0.72	0.02	-0.06	<0.05	0.82	0.11	-0.04	0.14	0.77	0.06	-0.05	0.09
Covariate (std.)	4.68	<0.001	0.26	<0.01	1.21	0.02	0.04	0.05	1.26	0.08	0.05	0.11
NAb titer 2° serotype (rel.)	0.70	0.01	-0.06	0.03	0.81	0.09	-0.04	0.11	0.75	0.04	-0.06	0.06
NAb titer 2° serotype (str.)	0.72	0.04	-0.06	0.07	0.84	0.22	-0.04	0.24	0.72	0.05	-0.07	0.07

[1]The odds ratio (OR) is change in the odds of symptomatic to inapparent DENV infection for each two-fold increase in NAb titer or one unit increase in the covariate value.

[2]The average marginal effect (AME) is the average effect of NAb titer or the covariate on the probability of symptomatic infection.

[3]Infection criteria: standard (std.), relaxed (rel.), stringent (str.).

[4]Covariate values (epidemic force, age, or years 1° to 2° infection) are only shown for the model including pre-infection NAb titer with standard infection criteria, but were included in all models.

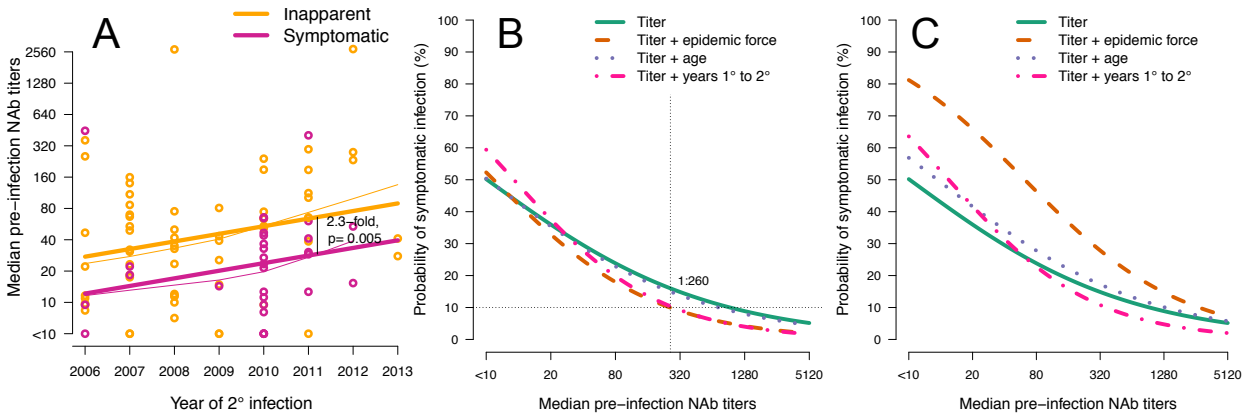

Figure 1. Higher median pre-infection NAb titers are associated with lower probability of symptomatic 2° DENV infection. (**A**) The year of 2° infection (2006-2013) plotted against the median pre-infection NAb titer for all children in subset 1, measured the year before 2° DENV infection. Local regression (thin lines, span=1) and linear regression (thick lines) are shown. (**B** and **C**) Logistic regression model predictions of the association between median pre-infection NAb titer and the probability of symptomatic infection (%). The predicted curves are drawn with covariate values set to the average value observed for each covariate (**B:** epidemic force=1.14, age=8.42, number of years between 1° and 2° infection=2.67) or the value observed in the 2009-2010 season (**C:** epidemic force=2.06, age=9.78, number of years between 1° and 2° infection=3.18).

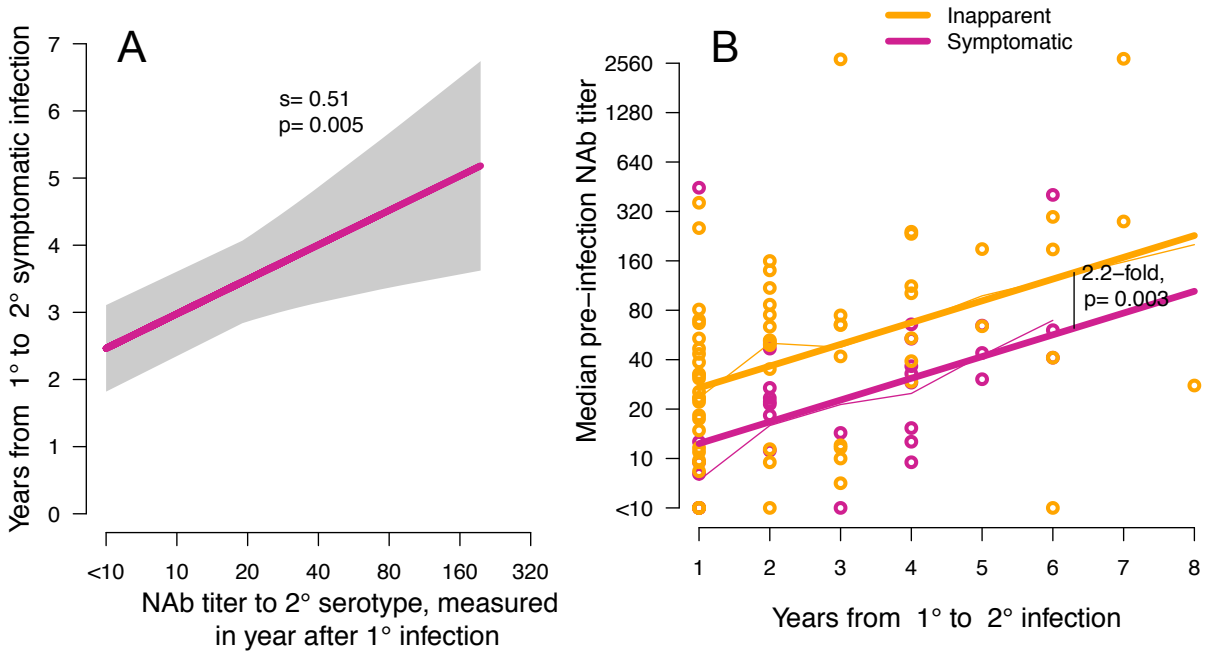

Figure 2. Higher pre-infection NAb titers are associated with delay in 2° symptomatic DENV infection. (**A**) The linear relationship between NAb titer to the 2° infecting serotype, measured immediately after 1° infection, and the number of years until symptomatic 2° infection; slope and p-value are shown. (**B**) The number of years between 1° and 2° infection (1-8) is plotted against the median pre-infection NAb titer for all children in subset 1, measured in the year before 2° infection. Local regression (thin lines, span=1) and linear regression (thick lines) are shown.

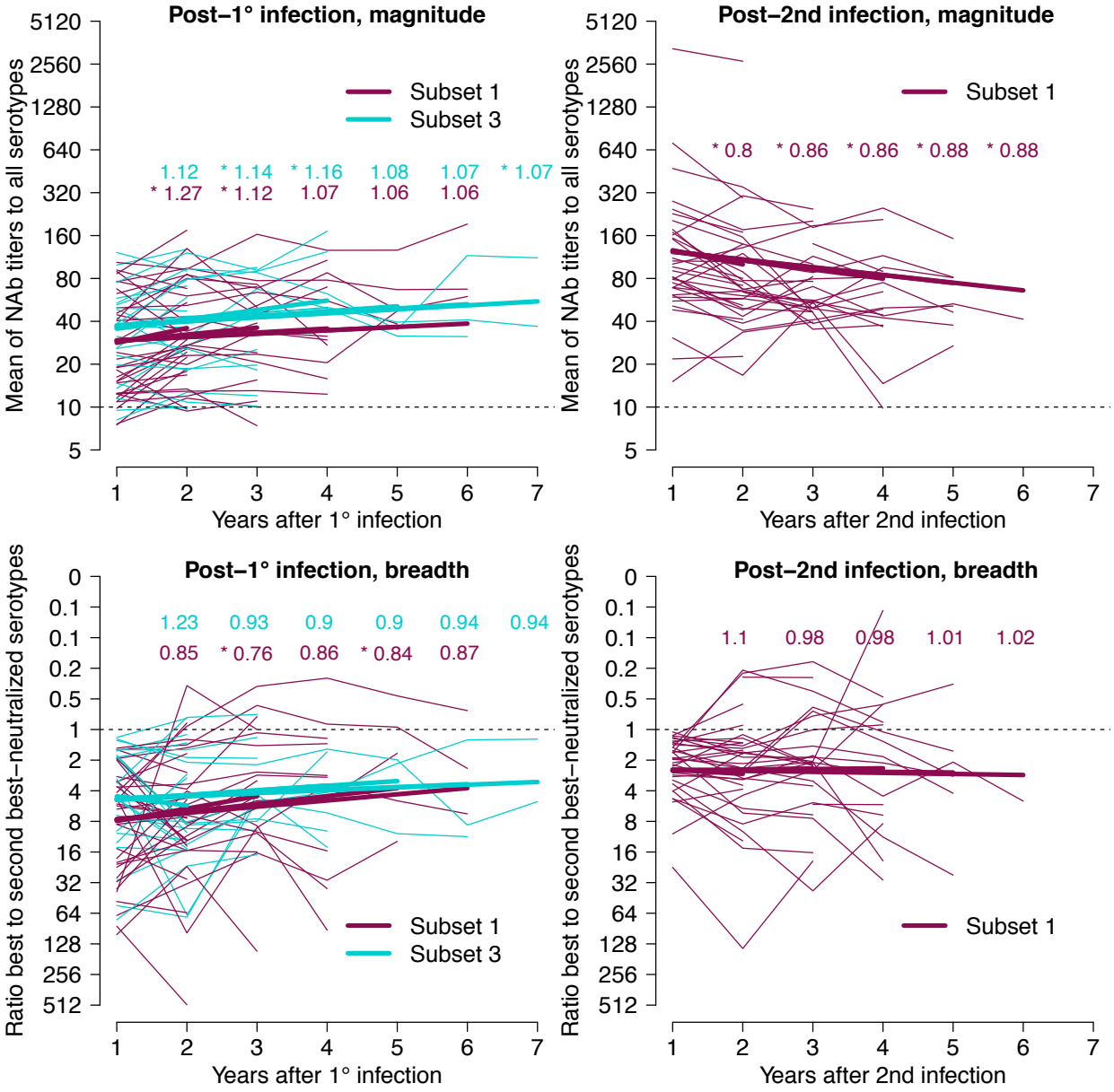

Figure 3. Dynamics of the magnitude and breadth of NAb titers after 1° and before subsequent infection (**A** and **B**), and between 2° infections (**C** and **D**), as measured with standard infection criteria. Plots show the number of years since previous infection against the magnitude (**A** and **C**) or breadth (**B** and **D**) of NAb titers. Thick lines showing the fold-change in the magnitude (values >1 are increasing) or breadth (values <1 are more cross-reactive) of NAb titers over time were estimated with linear regression. Fold-change estimates (see SI Appendix, Table S9) are indicated above regression lines, * indicates statistical significance at $p<0.05$.

Supplement to:

Neutralizing antibody titers against dengue virus correlate with protection from symptomatic infection in a longitudinal cohort

Leah C. Katzelnick, Magelda Montoya, Lionel Gresh, Angel Balmaseda, Eva Harris

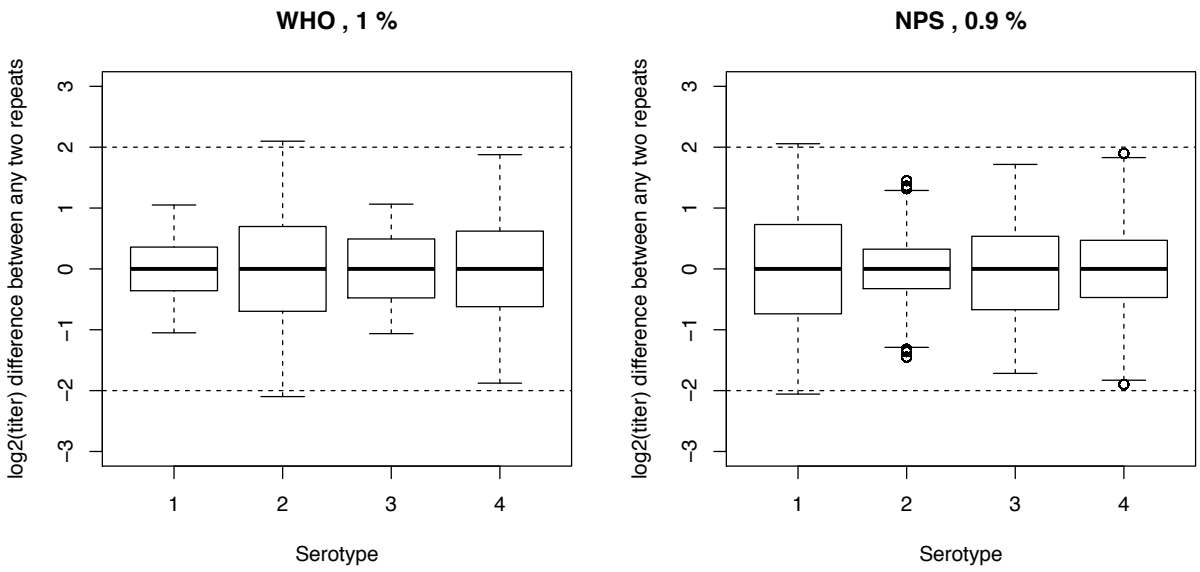

Figure S1. Positive control reproducibility of two polyvalent serum samples against RVPs representing DENV1-4. The positive controls titrated in ten independent experiments had standard deviations ranging from 0.38-0.78 $\log_2$, less than 2-fold deviation. Figures show 10,000 random comparisons of the $\log_2$ titer differences between any two titrations of the same serum and virus conducted on different days. Values above 2 indicate $\geq$4-fold increase between time points. **Left**, World Health Organization (WHO) polyvalent reference antisera, **Right**, Nicaraguan Polyvalent Sera (NPS). The percentage listed in the title indicates the percent of all titer comparisons that would include at least one of the four serotypes having a $\geq$4-fold increase, leading to erroneous identification of an infection.

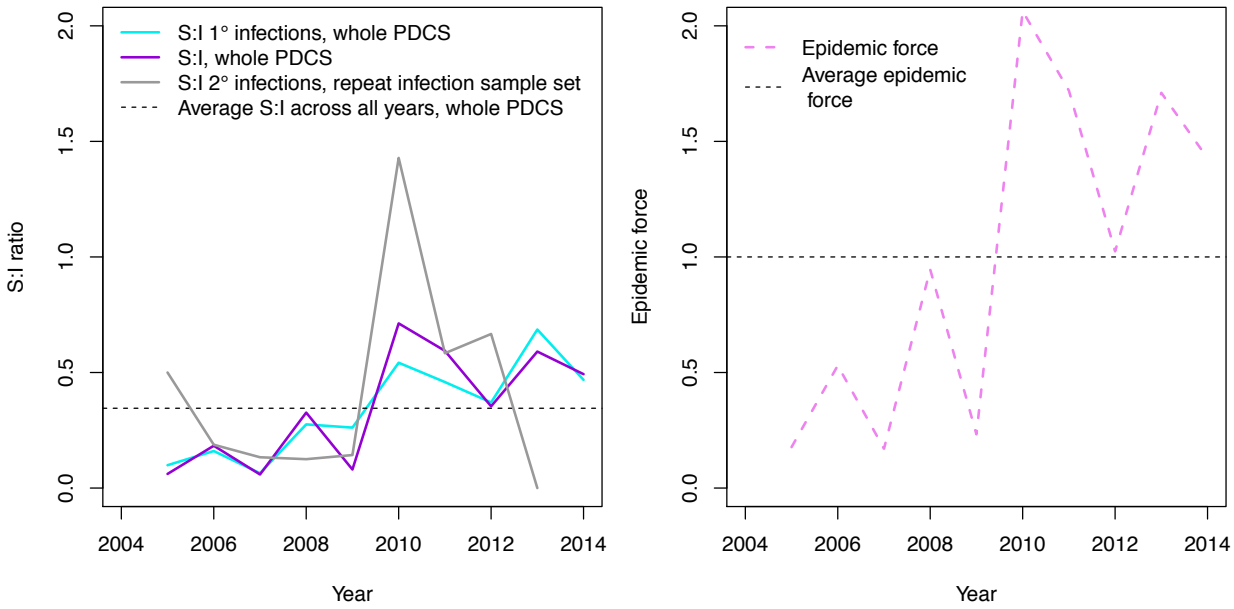

Figure S2. Method of estimating the epidemic force. (**Left**) Comparison of annual symptomatic to inapparent infection ratios (S:I ratio) for 1° infections (blue) or all infections (purple) in the full Nicaraguan Pediatric Dengue Cohort Study (PDCS), or 2° infections (grey) in the repeat infection sample set. The horizontal line (dotted black) indicates the average S:I ratio considering all infections in the whole PDCS over all years. (**Right**) The epidemic force, which is the annual S:I infection ratio for the whole PDCS divided by the average S:I ratio across all years of the whole PDCS (dotted pink line). The horizontal line (dotted black) indicates the average epidemic force, equal to 1.

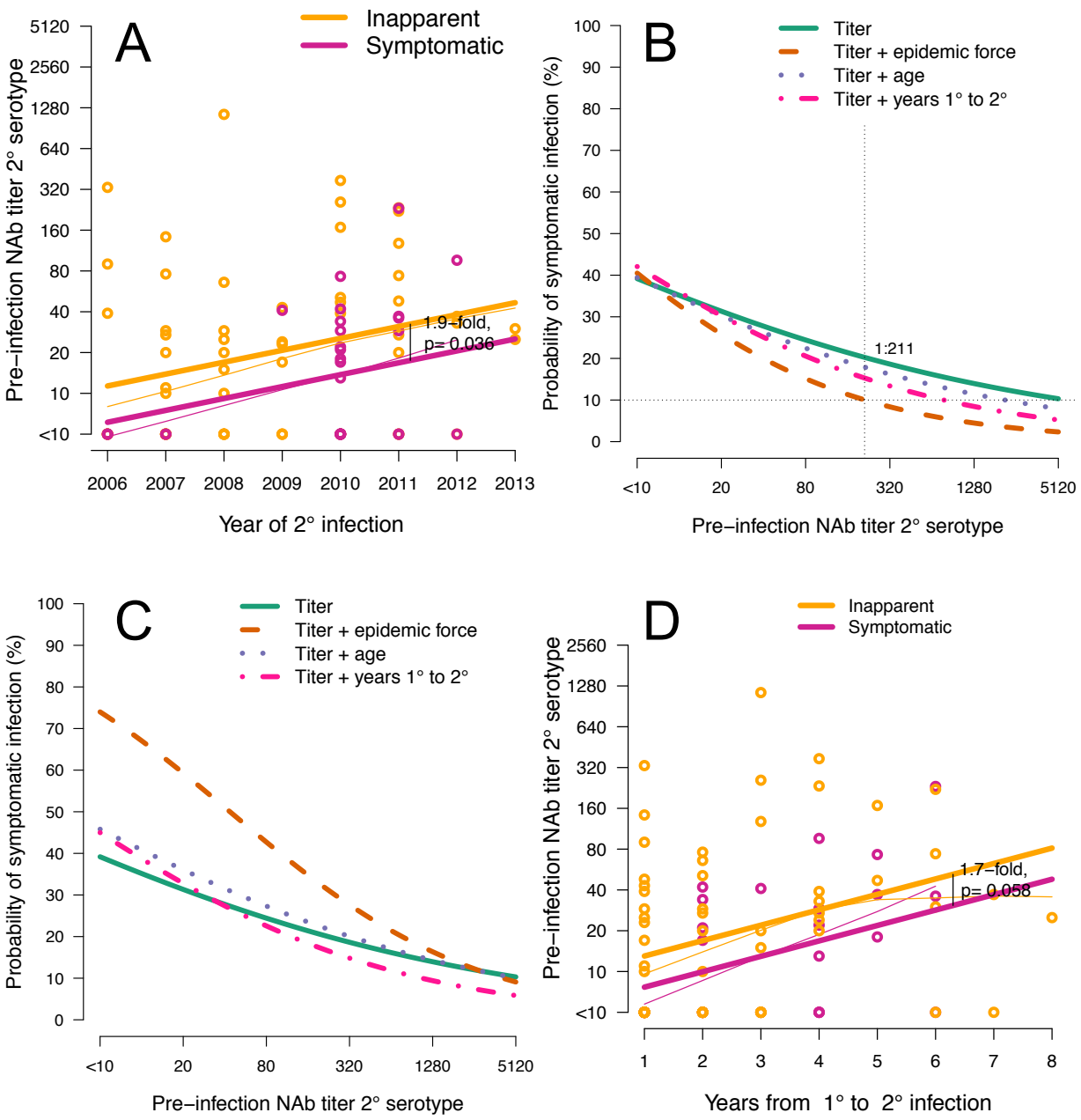

Figure S3. Higher pre-infection NAb titers to the 2° serotype are associated with lower probability of symptomatic 2° DENV infection. (**A**) The year of 2° infection (2006-2013) plotted against the pre-infection NAb titer to the 2° serotype for all children in subset 1, measured the year before 2° DENV infection. Local regression (thin lines, span=1) and linear regression (thick lines) are shown. (**B** and **C**) Logistic regression model predictions of the association between pre-infection NAb titer to the 2° serotype and the probability of symptomatic infection (%). The predicted curves are drawn with covariate values set to the average value observed for each covariate (**B:** epidemic force=1.14, age=8.42, number of years between 1° and 2° infection=2.67) or the value observed in the 2009-2010 season (**C:** epidemic force=2.06, age=9.78, number of years between 1° and 2° infection=3.18). (**D**) The number of years between

1° and 2° infection (1-8) is plotted against the pre-infection NAb titer to the 2° serotype for all children in subset 1, measured in the year before 2° infection. Local regression (thin lines, span=1) and linear regression (thick lines) are shown.

Table S1. Demographic characteristics and infection information in the repeat infection sample set.

	Subset 1[1] (n=69)		Subset 2[2] (n=19)		Subset 3[3] (n=24)		Full set (n=112)	
	Mean	*sd*	*Mean*	*sd*	*Mean*	*sd*	*Mean*	*sd*
Age at first infection[4]	5.99	2.53	4.84	2.12	7.83	2.99	6.19	2.72
Year of first infection[4]	2006-07	1.48	2004-05	1.02	2007-08	1.87	2006-07	1.82
Percent female (%)	46		37		46		45	

[1]Subset 1: children who entered the study DENV-naïve and had ≥2 infections (n=69).

[2]Subset 2: children who entered the study DENV-immune and had ≥1 infection (n=19).

[3]Subset 3: children who entered the study DENV-naïve and had only one infection (n=24).

[4]If immune at study enrollment (subset 2), the year of entry into the PDCS is used instead of the year of 1° infection.

Table S2. Number of 2° infections identified using different criteria for inapparent infections.

	Subset 1		Subset 2		Subset 3		Full set	
Infection criteria	*S*[1]	*I*	*S*	*I*	*S*	*I*	*S*	*I*
Standard[2]	32	66	6	21			38	87
Relaxed[3]	32	77	6	21		4	38	102
Stringent[4]	32	49	6	21			38	70

[1]Symptomatic infections are denoted S, inapparent infections, I.

[2]Standard infection criteria: a ≥4-fold increase to a DENV serotype an individual has not been infected with previously and to which they do not have a later symptomatic DENV infection.

[3]Relaxed infection criteria: any ≥4-fold increase in NAb titer.

[4]Stringent infection criteria: a ≥4-fold increase to a DENV serotype an individual has not been infected with previously and to which they do not have a later symptomatic DENV infection, plus an overall increase in median NAb titers.

Table S3. Relationship between pre-infection NAb titers and likelihood of symptomatic 2° infection (subset 1).

	Single logistic regression (subset 1)			
	NAb titer			
Variable (Definition of infection)	*OR*[1]	*p*	*AME*[2]	*p*
Median NAb titer (standard)	0.75	0.03	-0.06	0.05
Median NAb titer (relaxed)	0.76	0.03	-0.05	0.05
Median NAb titer (stringent)	0.82	0.18	-0.05	0.20
NAb titer 1° serotype (standard)	0.93	0.50	-0.02	0.50
NAb titer 2° serotype (standard)	0.84	0.16	-0.04	0.18
NAb titer 2° serotype (relaxed)	0.83	0.13	-0.04	0.15
NAb titer 2° serotype (stringent)	0.87	0.29	-0.03	0.30

[1]The odds ratio (OR) is change in the odds of symptomatic to inapparent infection for each 2-fold increase in NAb titer.

[2]The average marginal effect (AME) is the average effect of NAb titer on the probability of symptomatic infection.

Table S4. Relationship between pre-infection NAb titers and likelihood of symptomatic 2° infection (subset 1), with epidemic force estimated from 1° infection S:I ratio in the whole PDCS.

	Multiple logistic regression model (subset 1)			
	NAb titer + epidemic force			
Variable (Definition of infection)	*OR*[1]	*p*	*AME*[2]	*p*
Median NAb titer (standard)	0.69	0.02	-0.06	0.04
Epidemic force[3] (standard)	6.48	<0.001	0.32	0.01
Median NAb titer (relaxed)	0.68	0.01	-0.06	0.03
Median NAb titer (stringent)	0.72	0.07	-0.06	0.10
NAb titer 1° serotype (standard)	0.99	0.95	-0.001	0.95
NAb titer 2° serotype (standard)	0.72	0.03	-0.06	0.05
Epidemic force[3] (standard)	7.08	<0.001	0.35	0.01
NAb titer 2° serotype (relaxed)	0.71	0.02	-0.06	0.04
NAb titer 2° serotype (stringent)	0.70	0.03	-0.06	0.06

[1]The odds ratio (OR) is change in the odds of symptomatic to inapparent DENV infection for each 2-fold increase in NAb titer or one unit increase in the epidemic force.

[2]The average marginal effect (AME) is the average effect of NAb titer or epidemic force on the probability of symptomatic infection.

[3]Epidemic force is defined here by the 1° infection S:I ratio in the whole PDCS. The estimate of epidemic force is only shown for the model including pre-infection NAb titer with standard infection criteria, but was included in all models.

Table S5. Cross-validation of the model "NAb titer + epidemic force" to predict the probability of symptomatic infection (%) for subset 1.

% S, predicted	*0-10*[1]	*10-20*	*20-30*	*30-40*	*40-50*	*50-60*	*60-70*	*70-100*
Median NAb titers								
% S, full set	5	33	7	23	45	44	100	80
% S, test set[2]	13	23	12	20	42	48	97	79
NAb titer 2° serotype								
% S, full set	7	16	25	23	25	83	71	73
% S, test set	13	21	11	24	34	69	72	68

[1]Percent of infections that were symptomatic for each predicted probability (0-10%, 10-20% etc.) of symptomatic infection (S).
[2]Test set: random 10% of individuals per cross-validation trial, 10,000 separate trials.

Table S6. Relationship between pre-infection NAb titers and likelihood of symptomatic 2° infection for subsets 1, 2, & 3, in model "Nab titer + epidemic force".

	Subset 1 & 2				Subset 1, 2 & 3[1]			
	NAb titer + epidemic force				NAb titer + epidemic force			
Variable (Definition of infection)	*OR*[2]	*p*	*AME*[3]	*p*	*OR*	*p*	*AME*	*p*
Median NAb titer (standard)	0.68	0.01	-0.06	0.03				
Epidemic force[4] (standard)	4.02	<0.001	0.23	<0.01				
Median NAb titer (relaxed)	0.67	0.01	-0.06	0.02	0.66	0.01	-0.06	0.02
Median NAb titer (stringent)	0.73	0.05	-0.06	0.07				
NAb titer 1° serotype (standard)	1.00	0.98	0.00	0.98				
NAb titer 2° serotype (standard)	0.70	0.01	-0.06	0.03				
Epidemic force[4] (standard)	3.91	<0.001	0.23	<0.01				
NAb titer 2° serotype (relaxed)	0.69	0.01	-0.06	0.02	0.69	0.01	-0.06	0.02
NAb titer 2° serotype (stringent)	0.70	0.02	-0.06	0.04				

[1]All the same as subset 1 & 2 except for relaxed criteria.

[2]The odds ratio (OR) is change in the odds of symptomatic to inapparent DENV infection for each 2-fold increase in NAb titer or one unit increase in the epidemic force.

[3]The average marginal effect (AME) is the average effect of NAb titer or epidemic force on the probability of symptomatic infection.

[4]The estimate of epidemic force is only shown for the model including pre-infection NAb titer with standard infection criteria, but was included in all models.

Table S7. Relationship between pre-infection NAb titer and likelihood of symptomatic 2° infection, controlling for paired combinations of covariates.

Models	*OR*[1]	*p*	*AME*[2]	*p*
Median NAb titer (standard)	0.67	0.01	-0.07	0.04
Epidemic force (standard)	4.10	<0.001	0.23	0.01
Age (standard)	1.05	0.63	0.01	0.63
Median NAb titer (standard)	0.65	0.02	-0.07	0.04
Epidemic force (standard)	4.12	<0.001	0.23	0.01
Years 1° to 2° infection (standard)	1.07	0.68	0.01	0.68
Median NAb titer (standard)	0.66	0.01	-0.08	0.03
Years 1° to 2° infection (standard)	1.27	0.15	0.05	0.17
Age (standard)	1.13	0.21	0.02	0.23
NAb titer 2° serotype (standard)	0.71	0.02	-0.06	0.05
Epidemic force (standard)	4.32	<0.001	0.25	0.01
Age (standard)	1.06	0.55	0.01	0.56
NAb titer 2° serotype (standard)	0.72	0.03	-0.06	0.06
Epidemic force (standard)	4.72	<0.001	0.26	0.01
Years 1° to 2° infection (standard)	0.99	0.96	0.00	0.96
NAb titer 2° serotype (standard)	0.78	0.08	-0.05	0.11
Years 1° to 2° infection (standard)	1.13	0.43	0.02	0.44
Age (standard)	1.17	0.09	0.03	0.12

[1]The odds ratio (OR) is change in the odds of symptomatic to inapparent DENV infection for each 2-fold increase in NAb titer or one unit increase in the covariate value.

[2]The average marginal effect (AME) is the average effect of NAb titer or the covariate on the probability of symptomatic infection.

Table S8. Relationship between pre-infection NAb titer and likelihood of symptomatic 2° infection, controlling epidemic force, 1° infection outcome, 1° infecting serotype, or 2° infecting serotype.

Models	*OR*[1]	*p*	*AME*[2]	*p*
Median NAb titer (standard)	0.68	0.03	-0.05	0.06
Epidemic force (standard)	5.68	<0.001	0.24	0.01
1° infection outcome (standard)	0.05	0.01	-0.33	<0.001
Median NAb titer (standard)	0.57	0.004	-0.08	0.02
Epidemic force (standard)	5.80	<0.001	0.26	0.01
1° infecting serotype[3] (standard)	0.67	0.49	-0.06	0.49
Median NAb titer (standard)	0.75	0.10	-0.04	0.13
Epidemic force (standard)	4.56	<0.001	0.22	0.01
2° infecting serotype (standard, DENV1, OR=1)				
DENV2	2.31	0.34	0.12	0.31
DENV3	1.90	0.37	0.10	0.37
DENV4	0.00	0.99	-0.35	<0.001
NAb titer 2° serotype (standard)	0.55	<0.01	-0.08	0.02
Epidemic force (standard)	9.20	<0.001	0.29	0.01
1° infection outcome (standard)	0.02	<0.01	-0.38	<0.001
NAb titer 2° serotype (standard)	0.72	0.03	-0.05	0.06
Epidemic force (standard)	5.19	<0.001	0.27	0.01
1° infecting serotype[3] (standard)	0.70	0.53	-0.06	0.53
NAb titer 2° serotype (standard)	0.61	0.01	-0.07	0.03
Epidemic force (standard)	6.59	<0.001	0.26	0.01
2° infecting serotype (standard, DENV1, OR=1)				
DENV2	2.26	0.36	0.11	0.34
DENV3	2.23	0.29	0.11	0.29
DENV4	0.00	0.99	-0.37	<0.001

[1]The odds ratio (OR) is change in the odds of symptomatic to inapparent DENV infection for each 2-fold increase in NAb titer or one unit increase in the covariate value.

[2]The average marginal effect (AME) is the average effect of NAb titer or the covariate on the probability of symptomatic infection.

[3]Effect of 1° DENV2 infection relative to 1° DENV1 infection (OR=1). There were not enough 1° DENV3 or DENV4 infections in the sample set for analysis.

Table S9. Trajectories of NAb titer magnitude and breadth in the years between DENV infections, as measured with standard infection criteria.

		1° to subsequent infection				Between 2° infections	
		Subset 1		Subset 3		Subset 1	
	Years	*Est*[1]	*p*	*Est*	*p*	*Est*	*p*
Fold-change in the magnitude of NAb titers per year[2]	2	1.27	0.01	1.12	0.20	0.80	<0.01
	3	1.12	0.02	1.14	0.02	0.86	<0.01
	4	1.07	0.08	1.16	<0.001	0.86	<0.001
	5	1.06	0.10	1.08	0.06	0.88	<0.001
	6	1.06	0.06	1.07	0.06	0.88	<0.001
	7			1.07	<0.05		
Fold-change in breadth of NAb titers per year[3]	2	0.85	0.51	1.23	0.41	1.10	0.49
	3	0.76	0.03	0.93	0.64	0.98	0.75
	4	0.86	0.12	0.90	0.34	0.98	0.81
	5	0.84	0.03	0.90	0.27	1.01	0.82
	6	0.87	0.06	0.94	0.45	1.02	0.69
	7			0.94	0.42		

[1]The fold-changes per year in NAb titer magnitude and breadth were estimated with linear regression (slope), controlling for each individual's initial NAb responses by estimating individual intercept values. Separate models were run for all individuals with at least 2 years, 3 years, etc. of follow-up.

[2]Magnitude was measured as the mean of NAb titers to the four serotypes. Fold-changes in NAb titer magnitude <1 are decaying over time; >1, increasing in magnitude; =1, stable.

[3]Breadth was measured as the ratio of the NAb titer to 1° infecting serotype and the best-neutralized heterologous serotype. Fold-changes in NAb titer breadth <1 are more cross-reactive over time; >1, more type-specific; =1, stable.