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

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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 comorbidities 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 preinfection 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 with 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 reexposure. These findings establish that anti-DENV NAb titers correlate with reduced probability of symptomatic DENV infection and provide insights into longitudinal characteristics of antibody-mediated immunity to DENV in an endemic setting.

dengue virus | protection | neutralizing antibodies | cohort study | Nicaragua

Dengue virus (DENV) is a mosquito-borne flavivirus that infects up to 390 million individuals each year (1). Although 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 2 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 subneutralizing 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, after 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 preinfection 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 transplacental 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 preinfection NAb titers and reduced risk of symptomatic secondary (2°) infection, defined as two or more 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 preinfection 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 preinfection 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.

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 preinfection 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 reexposure. 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.

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The authors declare no conflict of interest.

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Results

Selection of the Repeat Infection Sample Set. The Nicaraguan Pediatric Dengue Cohort Study (PDCS; 2004 to 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 before 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 by using Inhibition ELISA (IE) on paired healthy annual samples processed side by side each year, defined as seroconversion (1° infections) or a fourfold or greater 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 one or more infections. From this group, we assembled the "repeat infection sample set" by randomly selecting 62 of 224 with two infections and 32 of 54 with three infections, as identified by either IE or symptomatic infection; 18 of 834 with one infection were also selected for comparison (n = 112 total) (9). Children in the repeat infection sample set were an average of 6.19 y old at the time of their first DENV infection, and 45% were female (*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 (*Materials and Methods*) (9).

Inapparent infections were classified by using the same criteria as reported previously (9): a fourfold or greater 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 serotypes at two time points to exhibit a fourfold or greater 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 (*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 of 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 two or more infections (n = 69); subset 2 entered the study DENV-immune and had one or more infections (n = 19); and subset 3 entered the study DENV-naïve and had only one infection (n = 24) (*SI Appendix*, Table S2).

We also identified the infecting serotype as described in *Materials and 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.

Preinfection NAb 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 preinfection NAb titers measured in the annual sample before the 2° infection and symptomatic vs. inapparent 2° DENV infections for subset 1, using single and multiple logistic regression. Preinfection 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 (*Materials and Methods* and *SI Appendix*, Fig. S2).

In all multiple logistic regression models, children with a higher median preinfection NAb titer were significantly less likely to experience symptomatic 2° infections (Fig. 1*A* and Table 1; *SI Appendix*, Tables S3 and 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 median preinfection NAb titer (Fig. 1*B*). Based on the model "median preinfection 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 preinfection NAb titer + epidemic force with cross-validation (*Materials and 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 (*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 preinfection NAb titer remained significant (*SI Appendix*, Table S6). In contrast, the preinfection NAb titer to the 1° infecting serotype was not significantly associated with infection outcome, suggesting that the heterotypic NAbs were providing protection (Table 1; *SI Appendix*, Tables S3, S4, and S6).

Alternative Criteria for Identifying Inapparent DENV Infections Do Not Change the Relationship Between Median Preinfection NAb Titer and Infection Outcome. We conducted sensitivity analyses to determine whether the protective effect of median preinfection NAb titer was robust to the criteria used to identify inapparent infections. By using "relaxed" infection criteria—defined as any fourfold or greater increase in NAb titer, thus allowing for reinfection with previous infecting serotypes—those in subset 1 with a higher median preinfection NAb titer were at reduced risk of symptomatic disease (Table 1; *SI Appendix*, Tables S2–S4). The relationship also remained when analyzing subsets 2 and 3 with the relaxed infection criteria (*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 more than onefold. Although the stringent criteria reduced the number of inapparent infections, the effect of median preinfection NAb titer on infection outcome remained (Table 1; *SI Appendix*, Tables S2–S4, and S6). We concluded that potential misclassification of inapparent infections would not substantively alter the measured relationship between preinfection NAb titer and protection.

Preinfection NAb Titer to the 2° Infecting Serotype Predicts Infection Outcome. We next tested whether the preinfection 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; *SI Appendix*, Tables S3, S4, and S6 and Fig. S3 *A–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; *SI Appendix*, S3, S4, and S6), and also performed well in cross-validation analyses (*SI Appendix*, 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

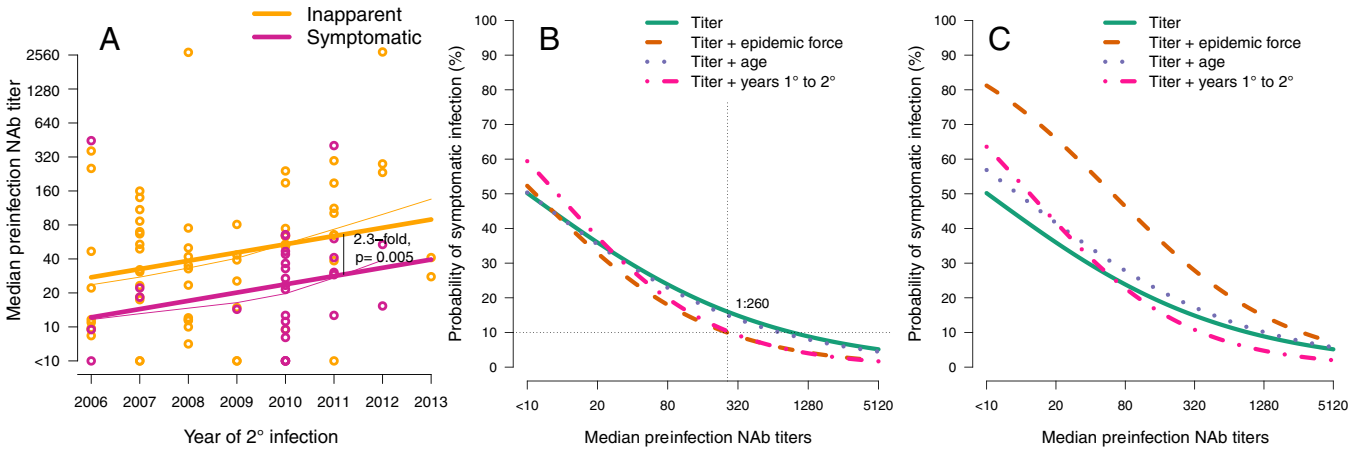

Fig. 1. Higher median preinfection NAb titers are associated with lower probability of symptomatic 2° DENV infection. (*A*) The year of 2° infection (2006–2013) plotted against the median preinfection NAb titer for all children in subset 1, measured the year before 2° DENV infection. Local regression (thin lines; span = 1) and linear regression (thick lines) are shown. (*B* and *C*) Logistic regression model predictions of the association between median preinfection 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).

of symptomatic 2° infection (Table 1). However, the logistic model with median preinfection 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 (*Materials and Methods* and *SI Appendix*, Table S7). DENV serotypes, genotypes, and strains differ in 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 (*SI Appendix*, Fig. S2). When each model covariate was set to the value observed in 2009–2010, the effect of median preinfection NAb titer on the probability of symptomatic infection was more striking (Fig. 1*C*) than that observed for average covariate values (Fig. 1*B*).

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 preinfection NAb titer + epidemic force. Children with symptomatic 1° infections were less likely to have symptomatic 2° infections independent of preinfection NAb titer (median or 2° serotype), suggesting that symptomatic 1° infection may modify subsequent disease risk in a way not fully measured by preinfection NAb titers. In contrast, we did not observe a significant relationship between either the 1° or the 2° infecting serotype and symptomatic 2° infection (*SI Appendix*, Table S8).

Higher Preinfection NAb 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.

Table 1. Preinfection 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 + years 1° to 2°			
Variable (infection criteria)	OR*	*P*	AME†	*P*	OR*	*P*	AME†	*P*	OR*	*P*	AME†	*P*
Median NAb titer (std.)	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 (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

Infection criteria are as follows: standard (std.), relaxed (rel.), and stringent (str.). Covariate values (epidemic force, age, or years 1° to 2° infection) are only shown for the model including preinfection NAb titer with standard infection criteria, but were included in all models.

*The odds ratio (OR) is change in the odds of symptomatic to inapparent DENV infection for each twofold increase in NAb titer or one unit increase in the covariate value.

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

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 against symptomatic infection. We found that every twofold higher NAb titer to the 2° infecting serotype, as measured immediately after 1° infection, provided a 6-month delay to symptomatic infection (Fig. 2*A*). We hypothesized that NAb titers would decay to low levels in all individuals by the year before 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 fourfold or greater increases in NAb titers between their 1° and 2° infections than those with earlier 2° infections ($P < 0.001$, linear regression). However, overall, those with 2° inapparent infections still consistently had higher preinfection NAb titers than those with 2° symptomatic infections (Fig. 2*B*; *SI Appendix*, Fig. S3*D*). These observations suggest that the increase in NAb titers may have been due to intermediate DENV exposures.

NAb Titers Increase in Magnitude and Breadth After 1° Infection. In the absence of reinfection 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 (two or more infections) and subset 3 (one infection) (*Materials and 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; *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, whereas 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; *SI Appendix*, Table S9).

Discussion

We have found that preinfection NAb titers reduce the risk of symptomatic DENV infection in a longitudinal dengue cohort. We observed that a higher median preinfection 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

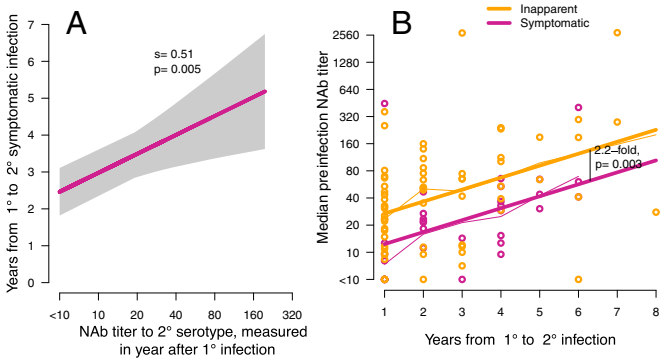

Fig. 2. Higher preinfection NAb titers are associated with delay in 2° symptomatic DENV infection. (*A*) The linear relationship between NAb titer to the 2° infecting serotype, measured immediately after 1° infection, and the number of years until symptomatic 2° infection; slope and *P* value are shown. (*B*) The number of years between 1° and 2° infection (1–8) is plotted against the median preinfection 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.

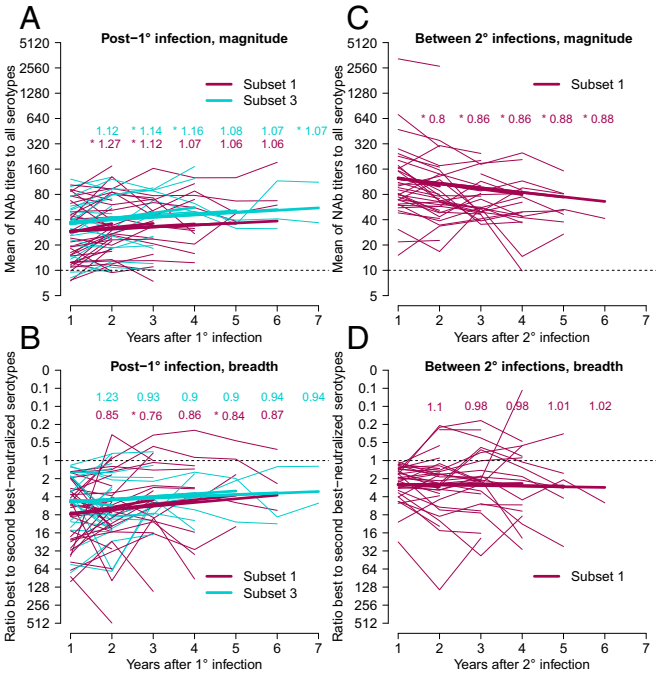

Fig. 3. Dynamics of the magnitude and breadth of NAb titers after 1° and before subsequent infection (*A* and *B*) and between 2° infections (*C* and *D*), as measured with standard infection criteria. Plots show the number of years since previous infection against the magnitude (*A* and *C*) or breadth (*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 (*SI Appendix*, Table S9) are indicated above regression lines. *$P < 0.05$.

NAbs determine protection, as others have proposed (7, 8, 12, 17, 19). The protective effect of preinfection 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, because 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 preinfection 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 preinfection NAb titers reduce the risk of severe disease (17), whereas 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 preinfection NAb titers than a comparable group of individuals likely, but not definitely, exposed to DENV but who did not become ill (18). However, because of 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 nonhuman cell substrates are not as biologically relevant (13). Our study fills a critical gap in

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the literature, establishing that higher levels of preinfection 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 by 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 at 2° infection, 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° infection, only epidemic force remained significant, suggesting that it was the major independent predictor of infection outcome besides preinfection NAb titer. Previous studies have found that the time between 1° and 2° infection 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 1° infections than DENV2 and DENV4; DENV2 causes the most symptomatic/severe 2° 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 reintroduction 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 preinfection 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 finding 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, by 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, because 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 nonendemic 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 after infection, but increased between 6 and 18 months after 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 by using standard infection criteria.

The frequency of boosts in NAb titer observed in the repeat infection sample set suggests that some may have been caused by reinfection with the 1° DENV serotype. This observation 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, because the dogma is that individuals have sterilizing immunity to all variants of the 1° infecting serotype (34). However, recent experimental studies in nonhuman primates demonstrated 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 plaque reduction neutralization test ($PRNT_{50}$), particularly if individuals had low NAb titers before the DENV2 challenge (35). Our findings suggest that DENV reexposure 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 reexposure 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 nonendemic 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 reexposure, including to homologous DENV serotypes, may help maintain these high levels of NAb titers over time.

Materials and Methods

Ethics Statement. The protocol for the Pediatric Dengue Cohort Study was reviewed and approved by the Institutional Review Boards (IRB) of the University of California, Berkeley, and the Nicaraguan Ministry of Health. Parents or legal guardians of all subjects provided written informed consent, and subjects 6 years of age and older provided assent.

Neutralization Titrations. NAb titers were measured as NT_{50} (relative to no antibody control) in a flow-cytometry-based assay by using human Raji-DC-SIGNR cells with RVPs representing the four DENV serotypes: DENV1, Western Pacific 74; DENV2, S16803; DENV3, CH53489; and 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 that 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 fourfold or greater 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 (greater than onefold, greater than twofold, or greater than fourfold) were similar to those estimated with >1.5-fold increase.

Epidemic Force. The epidemic force was estimated as the S:I ratio of 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 (*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 (*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 by using R (Version 3.1.3). We used logistic regression to test the association between median preinfection 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 [which allows comparison between models (37)], odds ratios (OR), or the probability of symptomatic infection [OR/(1 + OR)]. We conducted cross-validation of the model preinfection 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 median preinfection 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 y, 3 y, etc. of follow-up.

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