

Dengue vaccine-induced CD8⁺ T cell immunity confers protection in the context of enhancing, interfering maternal antibodies

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Declining levels of maternal antibodies were shown to sensitize infants born to dengue-immune mothers to severe disease during primary infection, through the process of antibody-dependent enhancement of infection (ADE). With the recent approval for human use of Sanofi-Pasteur's chimeric dengue vaccine CYD-TDV and several vaccine candidates in clinical development, the scenario of infants born to vaccinated mothers has become a reality. This raises 2 questions: will declining levels of maternal vaccine-induced antibodies cause ADE; and, will maternal antibodies interfere with vaccination efficacy in the infant? To address these questions, the above scenario was modeled in mice. Type I IFN-deficient female mice were immunized with live attenuated DENV2 PDK53, the core component of the tetravalent DENVax candidate currently under clinical development. Pups born to PDK53-immunized dams acquired maternal antibodies that strongly neutralized parental strain 16681, but not the heterologous DENV2 strain D2Y98P-PP1, and instead caused ADE during primary infection with this strain. Furthermore, pups failed to seroconvert after PDK53 vaccination, owing to maternal antibody interference. However, a cross-protective multifunctional CD8⁺ T cell response did develop. Thus, our work advocates for the development of dengue vaccine candidates that induce protective CD8⁺ T cells despite the presence of enhancing, interfering maternal antibodies.

Introduction

Dengue fever is the most prevalent arthropod-borne viral disease, with more than 200 million infections occurring worldwide each year (1). The etiological agent, dengue virus (DENV), exists as 4 antigenically distinct serotypes (types 1–4) that diverge substantially at the protein level such that infection by one serotype does not confer adequate protection to another, thereby giving rise to the possibility of reinfections by heterotypic viruses. Furthermore, preexisting immunity against DENV may place individuals at risk of developing severe forms of the disease, namely dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS) (2–5). Cross-reactive, nonneutralizing or subneutralizing levels of antibodies are believed to facilitate DENV infection of Fcγ receptor-expressing cells, thereby resulting in increased virus output that correlates with severe disease (6). Historically, epidemiological studies of infants born to dengue-immune mothers identified waning levels of maternal antibodies as a risk factor for severe dengue disease during a primary infection (5). We previously reported mouse models of maternal antibody-mediated enhancement of disease severity in AG129 (IFNα/β/γ R^{-/-}) and A129 (IFNα/β R^{-/-}) mice (7, 8). The use of IFN-deficient mouse strains is necessary to circumvent the natural resistance of wild-type mice to DENV infection (9). In addition to this antibody-dependent enhancement (ADE) of disease, the original antigenic sin (OAS) hypothesis has been proposed to play a role in disease severity. OAS involves cross-reactive memory T cells generated during a primary infection that preferentially activate and proliferate over naive T cells upon heterotypic reinfection. However, these reactivated memory T cells display suboptimal killing and altered cytokine profile with increased secretion of proinflammatory cytokines that may contribute to the vascular leak symptom of DHF/DSS (2–4).

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Despite decades of research, our understanding of the complexity of the immune response to DENV infection remains incomplete. As most vaccines do, dengue vaccines rely on antibodies to achieve protection (10). Strong evidence supports a role for neutralizing antibodies and the 50% neutralizing (PRNT₅₀) titer is widely adopted as a surrogate marker for vaccine efficacy (11–13). Although the presence of neutralizing antibodies may be associated with a lower risk of disease, a clear correlation between a defined level of neutralization and protection from any serotype remains to be established (14, 15). In particular, CYD-TDV (Dengvaxia), the first human-approved DENV vaccine, elicited generally high neutralizing titers in vaccinees but the overall protective efficacy across all 4 DENV serotypes was only 60.3% (16). Altogether, the available data suggest that the PRNT₅₀ titer alone does not adequately predict vaccine efficacy. This has led the dengue scientific community to consider the role of T cells in protection against DENV. Recent evidence from mouse and human studies suggests indeed that T cells, particularly the CD8⁺ subset, may be important mediators of protection (17–22). Greater appreciation of the importance of T cells is also evident from recent vaccine developments that focus on the assessment of T cell activity in vaccinated individuals and animals (23–26).

With the approval of CYD-TDV for human use and a few other vaccine candidates in advanced stages of clinical development (27), the scenario of infants acquiring maternal vaccine-induced antibodies will soon become a reality. Like the maternal antibodies generated by natural DENV infection, maternal vaccine-induced antibodies may also provide pan-serotype protection to the infant during the first few months after birth, followed by a window of enhanced susceptibility to severe dengue owing to the natural decay of antibodies to subneutralizing, enhancing levels. Although CYD-TDV is not recommended for children below the age of 9, it is likely that the next generation of dengue vaccines will be able to cover not only adults but also infants and young children, who represent a major population at risk of developing severe dengue in many countries (28). It is thus conceivable that maternal vaccine-induced antibodies may interfere with vaccination efficacy in the infant and thus limit protection, as previously seen with other vaccines (29). To investigate these areas of concern, we modeled the above scenario in mice. Type I IFN receptor-deficient A129 mice were used in this study to allow replication of the live attenuated DENV2 vaccine candidate PDK53. Although immunocompromised, the A129 mouse strain retains an intact adaptive immune system and IFN- γ signaling pathway, thus enabling the study of antibody and T cell responses. The vaccine candidate, PDK53, was derived from the wild-type 16681 DENV2 strain through serial passage in primary dog kidney cells and is the principal component of the tetravalent DENVax vaccine currently under clinical development (30). The role of maternal PDK53-induced antibodies in disease enhancement was investigated in the context of a heterologous infection. In addition, the protective potential of PDK53 vaccination of pups born to PDK53-immunized dams was examined.

Results

Maternal PDK53-induced antibodies protected pups against parental DENV2 strain 16681 but not against heterologous DENV2 strain D2Y98P-PP1. Adult A129 mice were infected with PDK53, and PRNT₅₀ titers against the parental DENV2 strain 16681 were monitored over time. Strong PRNT₅₀ titers were detected 3 weeks after vaccination and remained high throughout the observation period of 21 weeks (Figure 1A and Supplemental Figure 1A; supplemental material available online with this article; <https://doi.org/10.1172/jci.insight.94500DS1>). Consistently, 3-week-old (3-wko) pups born to PDK53-immunized dams also displayed high levels of neutralizing antibodies, unlike pups born to DENV-naive dams, confirming the presence of maternal DENV2 IgG antibodies (Figure 1B and Supplemental Figure 1B). By 6 and 9 weeks of age, PRNT₅₀ titers in pups born to immunized dams then declined, reflecting the natural decay of maternal antibodies over time (Figure 1B).

The ability of maternal antibodies to protect pups from infection was investigated by challenging 3-wko pups born to PDK53-immunized or nonimmunized (DENV-naive) dams with either the parental strain 16681 or the heterologous DENV2 strain D2Y98P-PP1 (31, 32). Infection with strain 16681 resulted in an asymptomatic transient viremia in pups born to DENV-naive dams (Figure 1C). In contrast, viremia was below the limit of detection in pups born to PDK53-immunized dams (Figure 1C), thus indicating protection by maternal antibodies and correlating with the strong PRNT₅₀ titers measured against strain 16681 in these 3-wko pups (Figure 1B). The heterologous strain D2Y98P-PP1 produced a symptomatic infection in pups born to naive dams — on day 4 after infection, all pups were symptomatic with a median clinical score of 3 (Figure 1D), as previously reported (8). Pups born to PDK53-immunized dams also developed symptoms and displayed a median clinical

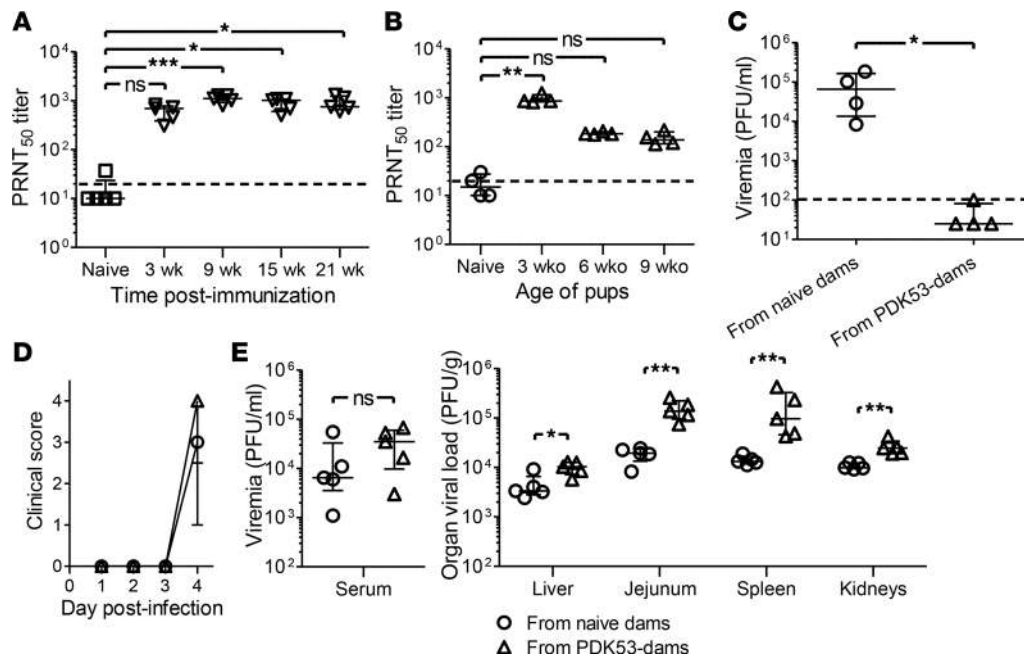


Figure 1. Neutralizing antibody titers in pups born to PDK53-immunized dams and disease outcome upon challenge with homologous or heterologous DENV2 strain. (A) Adult A129 mice ($n = 5$) were immunized with PDK53, and PRNT₅₀ titers against strain 16681 were monitored at the indicated time points. Naive controls (8 wko) were age matched to the first time point. Each data point represents 1 mouse; short horizontal lines represent medians and interquartile ranges. Limit of detection is represented by the horizontal dashed line. (B) PRNT₅₀ titers of pups against strain 16681. Pups ($n = 4$) born to PDK53-immunized dams were monitored at the indicated ages; age-matched pups born to naive dams served as controls. (C) 16681 viremia. Three-wko pups ($n = 4$) born to PDK53-immunized or naive dams were infected with 10⁷ PFU of 16681. Viremia was assessed by plaque assay at day 2 after infection. (D) Clinical scores of 3-wko pups born to PDK53-immunized or naive dams following 10⁶ PFU D2Y98P-PP1 challenge. 0, no observable symptoms; 1, ruffled fur; 2, diarrhea; 3, hunching; 4, severe hunching, both eyes shut, lethargy. (E) D2Y98P-PP1 viremia and organ viral loads at day 4 after infection. Medians and interquartile ranges are shown. PRNT₅₀ titers of immune sera were compared using Kruskal-Wallis test; remaining comparisons were done using Mann-Whitney test. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; ns, $P > 0.05$. Data are representative of 2 independent experiments.

score of 4 (Figure 1D), thus suggesting failure of maternal antibodies to protect against D2Y98P-PP1. Furthermore, significantly higher viral loads were measured in the liver, jejunum, spleen, and kidneys from pups born to PDK53-immunized dams compared with pups born to naive dams (Figure 1E). Altogether, the data indicated that pups born to PDK53-dams were protected from homologous 16681 challenge but experienced ADE upon challenge with heterologous DENV2 strain D2Y98P-PP1.

Comparative analysis of the envelope protein sequence to understand the lack of cross-protection by PDK53 immune serum. To investigate the lack of cross-protection observed in pups born to PDK53-immunized dams, the in vitro neutralizing activity of PDK53 immune serum was assessed against D2Y98P-PP1 virus. In both adult mice vaccinated with PDK53 and pups born to PDK53-immunized dams, PRNT₅₀ titers against the heterologous D2Y98P-PP1 strain (Figure 2, A and B, and Supplemental Figure 1, A and B) were clearly lower than the titers measured against the parental strain 16681 (Figure 1, A and B). Interestingly, comparable IgG titers were obtained when 16681 or D2Y98P-PP1 virus was used as coating antigen for indirect ELISA (Figure 2C), indicating that reduction in neutralizing activity against D2Y98P-PP1 was not due to an overt reduction in binding avidity.

The envelope (E) protein represents the major target for neutralizing antibodies — sequence analysis may help to explain the differences in PRNT₅₀ titers. E protein sequences from PDK53 and 16681 strains are completely conserved, whereas sequences from PDK53 and D2Y98P-PP1 are 98% identical (Supplemental Figure 2), suggesting that mutation of a small number of residues is sufficient to impair neutralization. It was previously shown that a substantial fraction of the murine neutralizing antibody response is directed at domain III of the E protein (EDIII) (33). Many strongly neutralizing monoclonal antibodies (mAbs) recognize epitopes located on the lateral ridge (e.g., 3H5-1) or the A-strand (e.g., MA1-27093) (34–36). EDIII sequences from PDK53/16681 and D2Y98P-PP1 were compared and found to differ by a single residue (N→S) at position 390 (Supplemental Figure 2), which was reported before as a variable position not critical for the binding of DENV2 type-specific or DENV complex-reactive antibodies (37). Based on

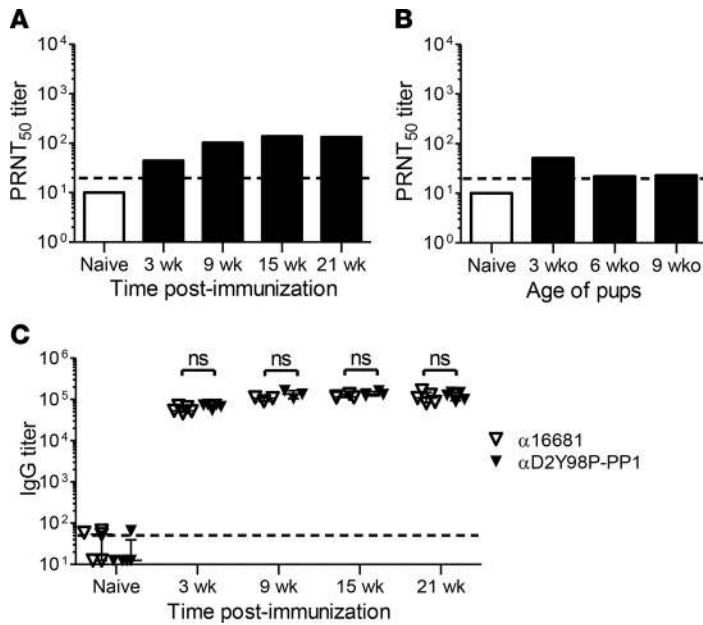


Figure 2. Neutralizing titers of PDK53-immune serum against D2Y98P-PP1. (A) PRNT₅₀ titers of adult A129 mice ($n = 5$) vaccinated with PDK53 against heterologous DENV2 strain D2Y98P-PP1. Sera were pooled at each time point. Limit of detection is indicated by horizontal dashed line. (B) PRNT₅₀ titers of pups ($n = 4$) born to PDK53-immunized dams against D2Y98P-PP1. Sera were pooled at each time point. (C) Serum IgG titers of adult PDK53-vaccinated mice against strains 16681 and D2Y98P-PP1. Each data point represents 1 mouse; short lines represent medians and interquartile ranges. Wilcoxon signed-rank test was used to compare anti-16681 (α 16681) and α D2Y98P-PP1 IgG titers at each time point. ns, $P > 0.05$. Data are representative of 2 independent experiments.

this information, we reasoned that 3H5-1 or MA1-27093 should neutralize both strains of DENV2 with similar efficacies. Unexpectedly, both mAbs were significantly more potent (32 and 10 times, respectively) at neutralizing strain 16681 than D2Y98P-PP1 (Table 1 and Supplemental Figure 1C). Given that the EDIII lateral ridge and A-strand sequences are completely conserved between strains 16681 and D2Y98P-PP1, it is unlikely that the amino acid difference at position 390 can alone account for the large disparity in PRNT₅₀ values obtained with both viruses. Hence, we speculate that sequence variation in other regions of the E protein may lead to a change in conformation of EDIII, which in turn affects binding and neutralizing activity of EDIII-targeting mAbs. Interestingly, the difference in PRNT₅₀ titers exhibited by PDK53 immune serum (5- to 17-fold) is somewhat similar to the differences observed with EDIII-specific mAbs, thus suggesting that structural and/or conformational differences in EDIII may possibly influence the neutralizing potency of PDK53 immune serum.

Pups born to PDK53-immunized dams did not seroconvert following PDK53 vaccination. Maternal antibodies have been reported to block antibody production in infants upon vaccination, a phenomenon known as maternal antibody interference (29). To test whether maternal PDK53-induced antibodies would interfere with the antibody response to PDK53 vaccination, 3-wko pups born to DENV-naive or to PDK53-vaccinated dams were vaccinated, or not, with PDK53 and blood was collected 1 day before (3 wko) and 19 days after (6 wko) vaccination to assess the PRNT₅₀ titers. Pups born to DENV-naive dams seroconverted from a prevaccination median PRNT₅₀ titer of less than 20 to a postvaccination titer of 254 against strain 16681 (Figure 3A). Nonvaccinated pups born to PDK53-vaccinated dams exhibited high levels of maternal antibodies at 3 weeks of age that declined thereafter (Figure 3A). Interestingly, PDK53-vaccinated pups born to PDK53-dams displayed PRNT₅₀ titers and decay pattern that were comparable to those of the nonvaccinated controls (Figure 3A), supporting an apparent lack of seroconversion that was possibly caused by maternal antibody interference. PRNT₅₀ titer stems from antibodies that target DENV structural

Table 1. Neutralizing ability of 3H5-1 and MA1-27093 mAb against 116681 and D2Y98P-PP1

mAb	PRNT ₅₀ value (μ g/ml)		Fold difference	P value
	Against 16681	Against D2Y98P-PP1		
3H5-1	0.2924 (0.1389)	9.494 (8.06)	32	0.0078
MA1-27093	0.9348 (0.5173)	9.725 (5.964)	10	0.0078

Antibodies were titrated from 1 mg/ml stocks against DENV2 strains 16681 and D2Y98P-PP1. Medians and interquartile ranges (in parentheses) were derived from 4 experiments. PRNT₅₀ values of each mAb against both DENV2 strains were compared using Wilcoxon signed-rank test.

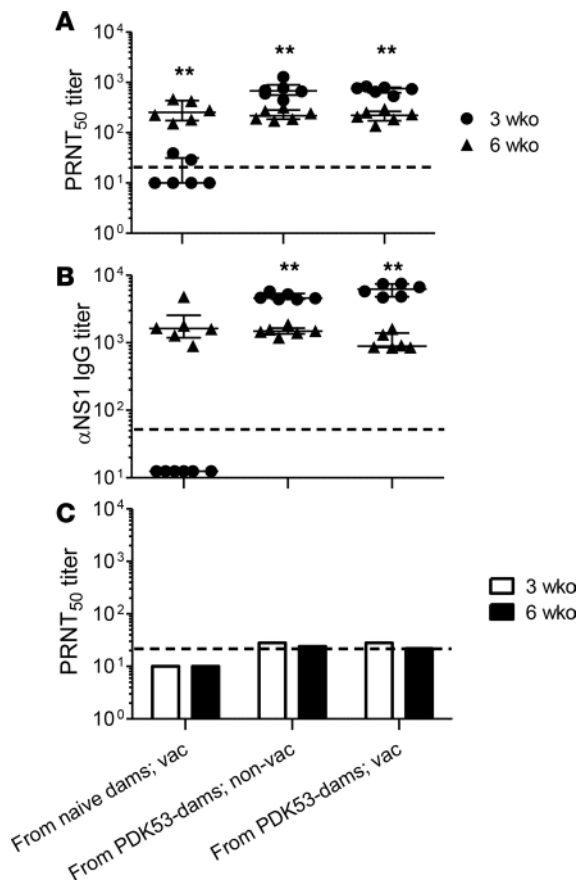


Figure 3. Antibody responses of pups immunized with PDK53. (A) PRNT₅₀ titers against strain 16681. Pups ($n = 6$) born to naive or to PDK53-immunized dams were immunized at 3 weeks of age; prevaccination and postvaccination titers were assessed at 3 and 6 weeks, respectively. Each data point represents 1 mouse; short lines represent medians and interquartile ranges. Mann-Whitney test was performed between 3- and 6-wko sera for each group. (B) DENV2 NS1-specific (α NS1) IgG titers. (C) PRNT₅₀ titers against strain D2Y98P-PP1. Sera were pooled at each time point. ** $P \leq 0.01$. Data are representative of 2 independent experiments.

proteins; to determine whether antibodies targeting the virus nonstructural proteins were similarly affected, serum IgG titers specific for DENV2 NS1 protein were measured by indirect ELISA. Pups born to DENV-naive dams developed clear anti-NS1 IgG responses 3 weeks after PDK53 vaccination (Figure 3B), indicating seroconversion as well as replication of PDK53 vaccine. Instead, nonvaccinated pups born to PDK53-vaccinated dams acquired maternal anti-NS1 IgG that decayed over time (Figure 3B). A similar pattern was observed in pups born to PDK53-vaccinated dams that were PDK53 vaccinated (Figure 3B), which further supported the hypothesis of maternal antibody interference. Finally, immune sera from all groups were found to poorly neutralize D2Y98P-PP1 (Figure 3C), consistent with earlier findings. In conclusion, PDK53 vaccination of pups born to PDK53-immune dams resulted in maternal antibody interference that prevented seroconversion, which eventually may impair vaccine efficacy.

PDK53 vaccination protected against D2Y98P-PP1 in the context of enhancing, interfering maternal antibodies. We have established that PDK53 antibodies were unable to protect against the heterologous D2Y98P-PP1 strain, likely due to E protein sequence divergence. We also demonstrated that maternal antibodies inhibit seroconversion upon PDK53 vaccination in pups born to PDK53-immune dams. However, we hypothesized that vaccination of these

pups with PDK53 may still induce T cell-mediated protection, which usually targets the more conserved nonstructural proteins (17, 18, 38, 39). It was indeed reported that the T cell response to vaccination was not impaired by maternal antibodies (29). Moreover, a protective role for DENV-specific T cells, particularly the CD8⁺ subset, was recently demonstrated in mice (17, 18, 20) and humans (19, 21).

To test this hypothesis, 3-wko pups born to DENV-naive or PDK53-immunized dams were vaccinated, or not, with PDK53 and subsequently challenged with D2Y98P-PP1 at 6 weeks of age (20 days after vaccination). The nonvaccinated groups developed clinical symptoms and were euthanized at day 4 after infection (Figure 4, A and B). In contrast, PDK53-vaccinated mice (born either to DENV-naive or PDK53-immunized dams) survived the D2Y98P-PP1 challenge without symptoms for 14 days (Figure 4, A and B), indicating successful induction of protective immunity. Assessment of the viral loads in sera and organ homogenates by plaque assay revealed the absence of detectable viral titers at day 4 after infection in all the vaccinated mice, which further confirmed the protective efficacy of PDK53 vaccination (Figure 4C). Among the nonvaccinated groups, higher viral loads were again observed in mice born to PDK53-immunized dams (Figure 4C), confirming enhancement of infection.

Altogether, the data indicated that PDK53 vaccination fully protected against D2Y98P-PP1 challenge despite the presence of enhancing, interfering antibodies, thus strongly suggesting the involvement of T cells in the mechanism of protection.

PDK53-induced protection against D2Y98P-PP1 was mediated by CD8⁺ T cells. To address the protective role of PDK53-induced T cells in vaccinated pups born to PDK53-immunized dams, adoptive transfer experiments were performed. Total CD4⁺ and CD8⁺ T cells were isolated from spleens and lymph nodes of 6- to 8-wko donor mice vaccinated, or not, with PDK53, and 1.5×10^7 CD4⁺ or CD8⁺ T cells were adoptively transferred to 5- to 7-wko recipient mice born to PDK53-immunized dams, followed by D2Y98P-PP1 challenge (10^7 PFU/mouse) 1 day later. Mice adoptively transferred with CD4⁺ or CD8⁺ T cells from nonvaccinated donors developed severe symptoms 4 days after infection and were euthanized (Figure 4, D and E). Similarly, mice transferred with CD4⁺ T cells from PDK53-vaccinated donors succumbed to the infection (Figure 4D). In contrast, mice that received CD8⁺ T cells from PDK53-vaccinated donors remained

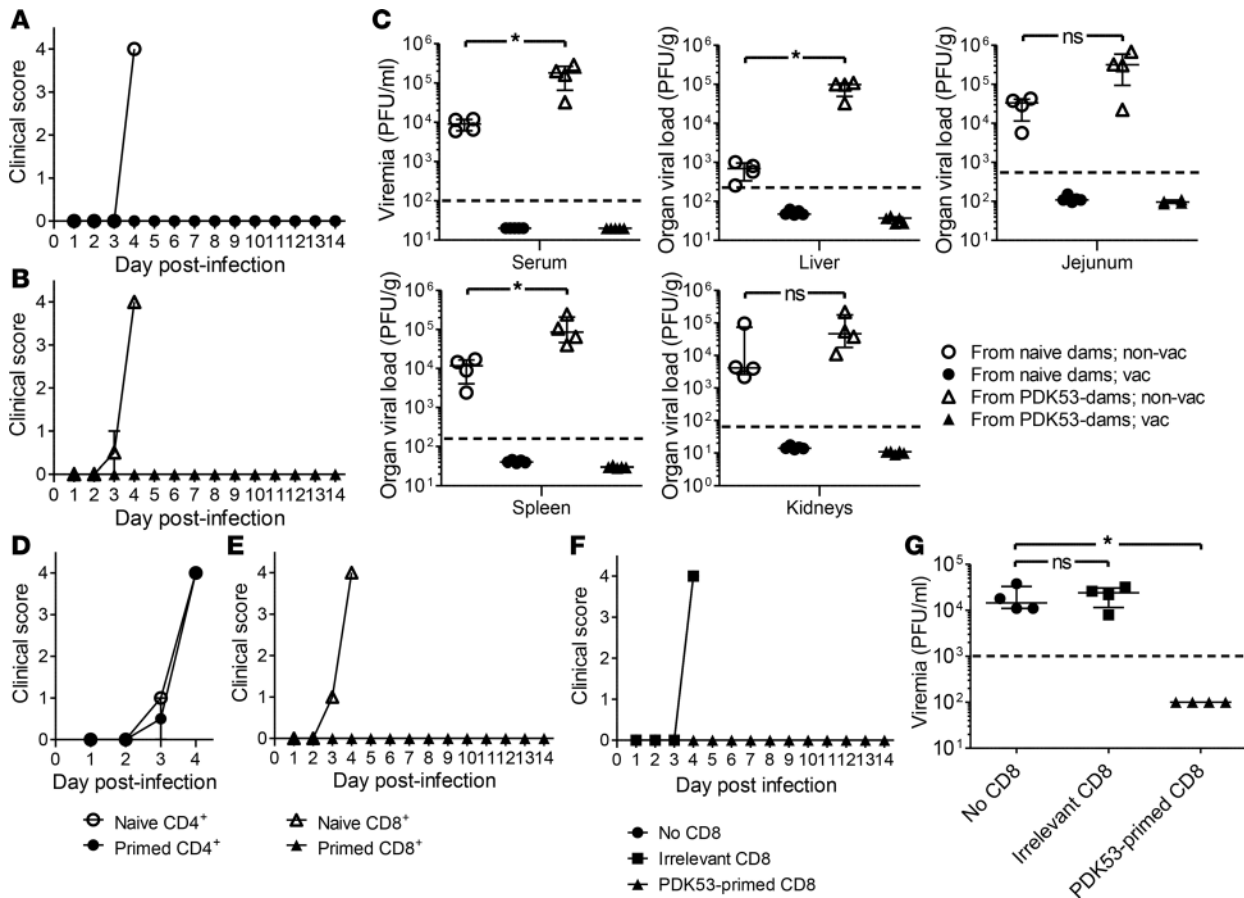
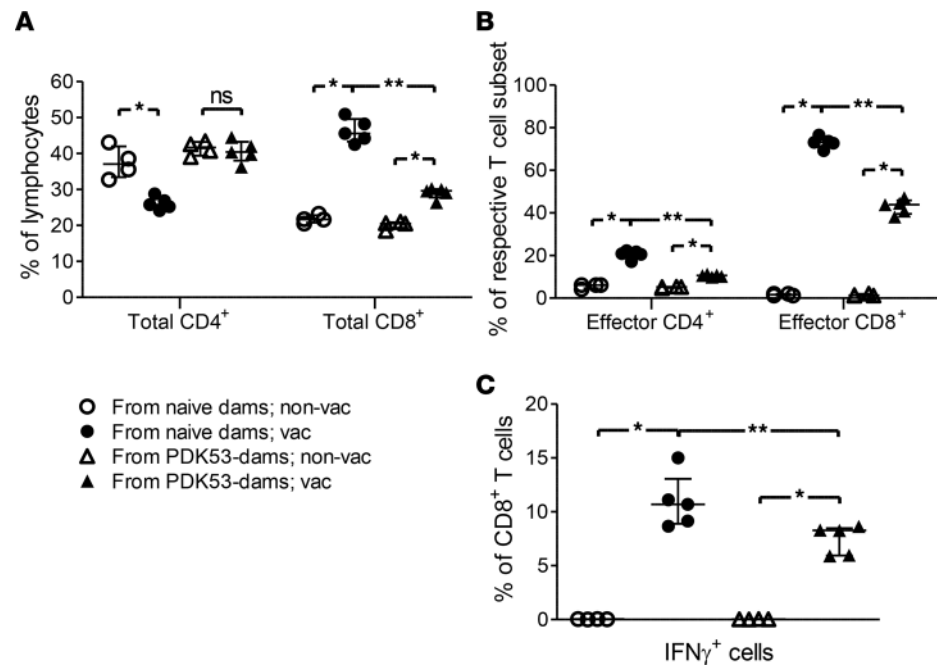


Figure 4. Disease outcome upon challenge with D2Y98P-PP1 of pups born to PDK53-immunized dams and vaccinated with PDK53 or passively transferred with CD4⁺ or CD8⁺ T cells. Three-wko A129 pups born to PDK53-immunized or naive dams were vaccinated, or not, with PDK53 and challenged at 6 weeks of age with 10^7 PFU of D2Y98P-PP1 strain. (A and B) Clinical scores. Euthanasia was performed at a clinical score of 4. Medians and interquartile ranges of 5–6 mice per group are shown. (C) D2Y98P-PP1 viremia and organ viral loads. Nominal values were assigned to viral loads below limit of detection. Mann-Whitney test was performed to compare viral loads between the nonvaccinated groups. (D and E) Five- to 7-wko mice born to PDK53-immune or DENV-naive dams were transferred with 1.5×10^7 naive or PDK53-primed CD4⁺ (D) or CD8⁺ (E) T cells and challenged with 10^7 PFU of D2Y98P-PP1 one day later. Medians and interquartile ranges of 7–8 (D) and 5 (E) mice per group are shown. Euthanasia was performed at a clinical score of 4. Data are representative of 2 independent experiments. (F) Clinical scores of 6-wko A129 mice ($n = 8$) born to PDK53-immunized dams, and adoptively transferred with 6×10^6 CD8⁺ T cells 1 day prior to D2Y98P-PP1 challenge (10^6 PFU). The CD8⁺ T cells transferred were either nonspecifically activated or PDK53-primed. (G) D2Y98P-PP1 viremia at day 4 after challenge ($n = 4$) in mice transferred with either nonspecifically activated CD8⁺ T cells or PDK53-primed CD8⁺ T cells. Kruskal-Wallis test with Dunn’s multiple comparison was performed. * $P \leq 0.05$; ns, $P > 0.05$. Data are representative of 2 independent experiments.

asymptomatic across the 14-day observation period (Figure 4E), thus demonstrating that PDK53-primed CD8⁺ T cells but not CD4⁺ T cells were sufficient to confer protection against D2Y98P-PP1 in pups born to PDK53-immunized dams.

To confirm that protection was mediated by virus-specific rather than nonspecifically activated CD8⁺ T cells, splenocytes were harvested from naive A129 mice and stimulated in vitro with anti-CD3 and anti-CD28 antibodies. Due to limited cell numbers, the adoptive transfer experiment was performed with 6×10^6 CD8⁺ T cells and mice were challenged with a lower dose (10^6 PFU) of D2Y98P-PP1 one day later (Figure 4, F and G). Mice transferred with nonspecifically activated CD8⁺ T cells, or not transferred at all, developed severe symptoms 4 days after challenge and were euthanized (Figure 4F). In contrast, mice adoptively transferred with PDK53-primed CD8⁺ T cells survived the challenge without symptoms for 14 days (Figure 4F). Consistently, high virus titers were measured in mice that were transferred with nonspecifically activated CD8⁺ T cells, or in no-transfer control mice, whereas mice that received PDK53-primed CD8⁺ T cells had no detectable viremia (Figure 4G). Altogether, these data indicated that protection against D2Y98P-PP1 challenge in PDK53-vaccinated pups born to PDK53-immunized dams was mediated by PDK53-specific CD8⁺ T cells.



PDK53 vaccination induced a substantial dengue-specific CD8⁺ T cell response. To characterize the T cell response induced by PDK53 vaccination, 3-wk pups born to DENV-naive or to PDK53-immunized dams were vaccinated, or not, with PDK53 and blood was collected 7 days later for analysis of peripheral blood mononuclear cells (PBMCs) by flow cytometry. A significant increase in median proportion of total CD8⁺ but not CD4⁺ T cells was seen in the vaccinated groups compared with their respective nonvaccinated controls (Figure 5A and Supplemental Figure 3A), suggesting proliferation of CD8⁺ T cells in response to PDK53 vaccination. A 2.1-fold increase in CD8⁺ T cells was seen in pups born to DENV-naive dams, whereas pups born to PDK53-immunized dams exhibited a 1.4-fold increase, suggesting possible dampening of the CD8⁺ T cell response due to the presence of maternal antibodies. The proportions of effector (CD44^{hi}CD62L^{lo}) CD4⁺ and CD8⁺ T cells were significantly elevated after vaccination (Figure 5B and Supplemental Figure 3B) but the magnitude of increase for CD8⁺ T cells (33- to 45-fold) was markedly greater than for CD4⁺ T cells (2.0- to 3.4-fold). In addition, the magnitude of increase in effector T cells was weaker in pups born to PDK53-vaccinated dams compared with pups born to naive dams (Figure 5B), again suggesting that the presence of maternal antibodies may limit the magnitude of T cell responses.

To study the DENV-specific CD8⁺ T cell responses, PBMCs from the different animal groups were stimulated with the NS4B₉₉₋₁₀₇ peptide, which contains a highly immunodominant CD8⁺ T cell epitope (17). IFN- γ production was detected only in CD8⁺ T cells from both PDK53-vaccinated groups (Figure 5C and Supplemental Figure 3C), thus indicating DENV specificity. Of note, these results imply that in mice born to immunized dams, the PDK53 strain replicated effectively despite the presence of neutralizing maternal antibodies. However, the median proportion of IFN- γ ⁺ CD8⁺ T cells in pups born to PDK53-immunized dams (8.3%) was slightly but significantly lower than in pups born to DENV-naive dams (10.7%), suggesting that viral replication was restricted by maternal antibodies.

A multifunctional CD8⁺ T cell response was triggered in PDK53-vaccinated pups upon challenge with D2Y98P-PP1. The T cell responses triggered upon D2Y98P challenge were characterized. Mice born to PDK53-immunized dams were vaccinated with PDK53 at 3 weeks of age and subsequently challenged with D2Y98P-PP1 at 6 weeks of age. Three days after challenge their spleens were harvested. The CD4⁺ and CD8⁺ T cell

populations were compared to the nonvaccinated–challenged controls and nonvaccinated–nonchallenged controls. Mice from the nonvaccinated–challenged group exhibited slight but significant reduction in total CD4⁺ and CD8⁺ T cell numbers (1.5- and 1.7-fold, respectively) after infection, compared with the nonvaccinated–nonchallenged group (Figure 6A), indicating a mild lymphopenia that has been previously reported in dengue patients (40) and mouse models of dengue (8, 31). In contrast, mice vaccinated with PDK53 displayed significantly higher numbers of CD4⁺ and CD8⁺ T cells compared with the nonvaccinated–challenged group (1.7- and 3.5-fold increase, respectively) (Figure 6A). Similar observations were made with the effector T cell populations, with a striking 84-fold increase in the number of effector CD8⁺ T cells in the vaccinated–challenged group compared with the nonvaccinated–challenged group (Figure 6B).

To assess DENV-specific CD4⁺ and CD8⁺ effector responses, splenocytes were incubated with DENV2 NS3_{198–212} and NS4B_{99–107} peptides, which contain immunodominant CD4⁺ and CD8⁺ T cell epitopes, respectively (17, 18), and expression of CD107a (degranulation marker), intracellular IFN- γ and TNF- α (Th1 cytokines), and IL-5 (Th2 cytokine) was examined by flow cytometry. Splenocytes from nonvaccinated–nonchallenged mice were used to define positive gates (Supplemental Figures 4 and 5); only responses significantly higher than background were taken into consideration. CD4⁺ T cells from the nonvaccinated–challenged group responded to peptide stimulation with IFN- γ production, whereas CD4⁺ T cells from the vaccinated–challenged group responded with IFN- γ and TNF- α production (Figure 6C and Supplemental Figure 4). Expression of IL-5 was not detected in any group. Altogether, these observations indicated a Th1-biased response, which is consistent with the primary effector function of CD4⁺ T cells during a viral infection (41, 42). Significantly higher proportions of responding CD4⁺ T cells were detected in splenocytes from vaccinated–challenged mice compared with nonvaccinated–challenged controls (Figure 6C), consistent with the greater number of effector cells measured (Figure 6B).

CD4⁺ T cells from the nonvaccinated–challenged and vaccinated–challenged groups were largely monofunctional (Figure 6, D and F), expressing either IFN- γ (median proportions of 0.13% and 0.69%, respectively) or CD107a (median proportions of 0.54% and 0.57%, respectively). Within the bifunctional subpopulation, cells were either CD107a⁺IFN- γ ⁺ (0.037% and 0.22%, respectively) or TNF- α ⁺IFN- γ ⁺ (0.002% and 0.028%, respectively). Finally, a tiny proportion of CD4⁺ T cells were found to be triple positive (CD107a⁺IFN- γ ⁺TNF- α ⁺; 0.015%) in the vaccinated–challenged group. Overall, PDK53 vaccination allowed a greater percentage of multifunctional CD4⁺ T cells after D2Y98P challenge (Figure 6F).

The proportion of CD8⁺ T cells that responded to peptide stimulation was markedly higher in vaccinated–challenged mice compared with nonvaccinated–challenged controls (Figure 6E). Within the multifunctional subpopulation, a striking 18.1% of CD8⁺ T cells from vaccinated mice were CD107a⁺IFN- γ ⁺; within the monofunctional subpopulation, cells mostly expressed CD107a (13.3%) followed by IFN- γ (2.23%). Like the CD4⁺ T cells, PDK53 vaccination also resulted in greater multifunctionality of the CD8⁺ T cells (Figure 6G). Since multifunctional T cells have been associated with improved control of infection (43), the high frequency of CD107a⁺IFN- γ ⁺ CD8⁺ T cells in the vaccinated–challenged group suggested a crucial role in mediating protection against D2Y98P-PP1. In particular, strong expression of degranulation marker CD107a indicated CD8⁺ T cell–mediated cytotoxicity to be a major mechanism of protection. On the other hand, low frequency of responding CD4⁺ T cells, and an even lower frequency of multifunctional cells, would suggest a limited role in DENV immunity.

To further demonstrate the critical role of cytotoxic CD8⁺ T cells in protection, PDK53-immunized mice were adoptively transferred with a mix of equal proportions of splenocytes harvested from naive mice and pulsed *in vitro* with either NS4B_{99–107} or with irrelevant ovalbumin (OVA) peptide (SIINFEKL). Each population was then labeled with a different concentration of CFSE for easy tracking. A marked reduction in the population of NS4B_{99–107}-pulsed cells compared with the OVA-pulsed population was observed (Figure 6H); such reduction was not seen in nonvaccinated control mice, which displayed similar proportions of both populations (Figure 6H). Therefore, these observations demonstrate the cytotoxic activity of PDK53-primed CD8⁺ T cells towards DENV-infected cells specifically, thus supporting a role for these CD8⁺ T cells in protection against D2Y98P-PP1.

Discussion

PDK53 is a well-characterized, highly immunogenic live attenuated DENV2 vaccine candidate shown to elicit moderate to high neutralizing titers in human volunteers and AG129 mice (44–50). In our study, a single dose of PDK53 induced in A129 mice high and sustained PRNT₅₀ titers against parental DENV2

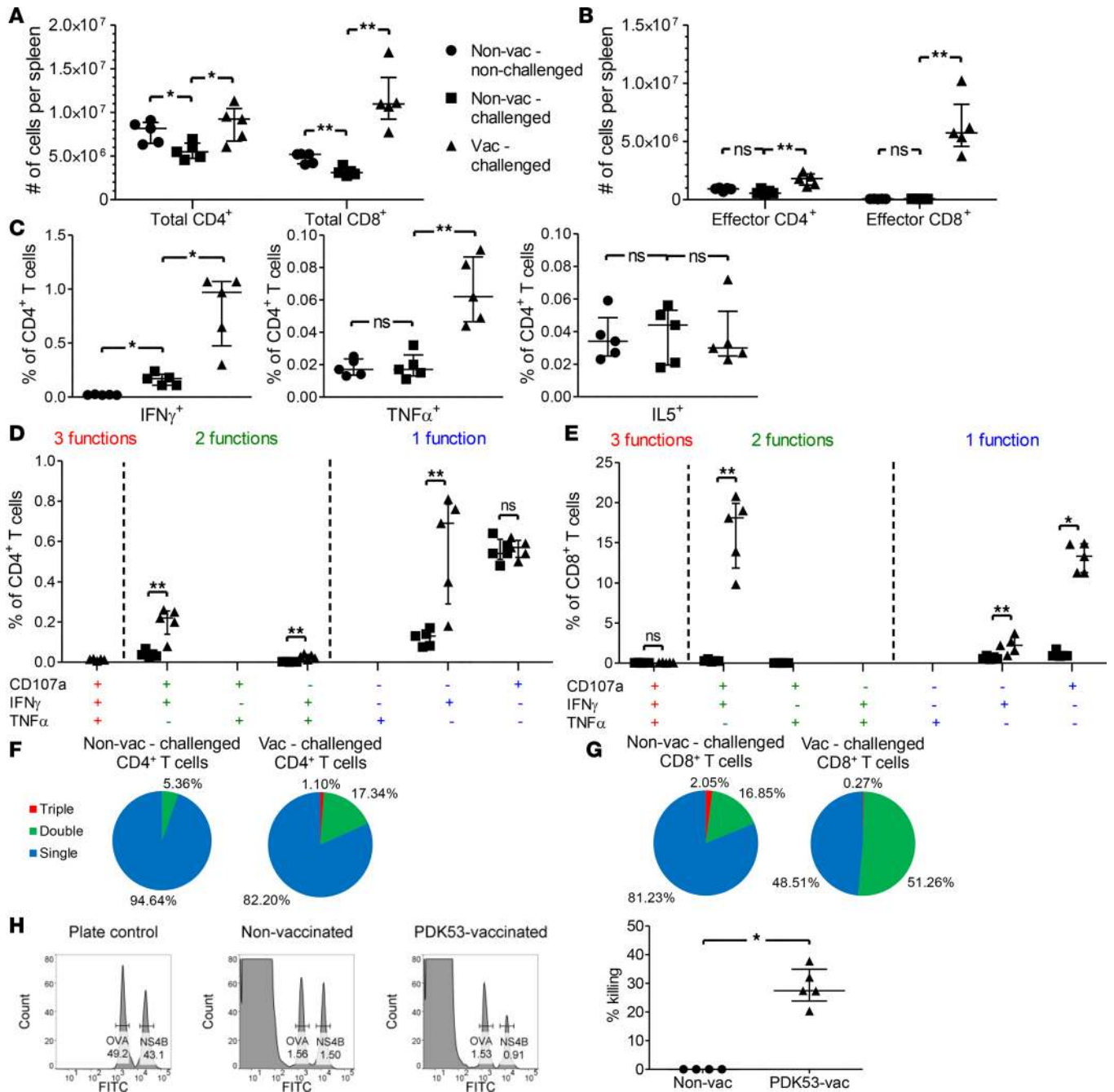


Figure 6. T cell responses upon D2Y98P-PP1 challenge in PDK53-vaccinated mice born to PDK53-immune dams. (A) Three-wko pups ($n = 5$) born to PDK53-immune dams were immunized, or not, with PDK53 and challenged with 10^7 PFU of D2Y98P-PP1 at 6 weeks of age. Proportions of splenic CD4⁺ and CD8⁺ T cells were determined by flow cytometry and converted to absolute numbers. (B) Effector CD4⁺ and CD8⁺ T cell numbers per spleen. (C) Proportions of CD4⁺ T cells expressing IFN- γ , TNF- α , or IL-5 following DENV2 peptide in vitro stimulation. (D and E) Effector functions of CD4⁺ and CD8⁺ T cells. Boolean gating was applied to identify all combinations of effector functions. Only responses significantly higher than background are shown. (F and G) Fractions of responding T cells with 1, 2, or 3 functions. (H) In vivo cytotoxicity assay. Three-wko mice born to PDK53-immune dams were immunized, or not, with PDK53 and adoptively transferred with a mix (in equal proportions) of differentially CFSE-labeled NS4B₉₉₋₁₀₇-pulsed or OVA-pulsed (SIINFEKL) splenocytes at 6 weeks of age. Mice were euthanized after 4 hours and their spleens were harvested for enumeration of peptide-pulsed cells by flow cytometry. Representative histograms are shown; numbers represent the proportions of peptide-pulsed, CFSE-labeled cells. Ratio of OVA-pulsed to NS4B₉₉₋₁₀₇-pulsed cells was calculated to derive the percentage killing: $(1 - [\text{plate control ratio}/\text{experimental ratio}]) \times 100$. Mann-Whitney test was performed. * $P \leq 0.05$; ** $P \leq 0.01$; ns, $P > 0.05$. Data are representative of 2 independent experiments.

strain 16681, in line with the reported immunogenicity of this vaccine candidate. Despite potent neutralization of 16681 virus, we showed that immune sera from PDK53-vaccinated A129 mice failed to effectively neutralize the heterologous DENV2 strain D2Y98P-PP1, suggesting that epitopes presented by D2Y98P-PP1 are significantly different from those in PDK53 or 16681. This finding appears to contradict the current consensus, which states that neutralizing epitopes are conserved within each serotype (36). Nevertheless, other studies looking at vaccine-induced neutralizing activity (51), mAb neutralizing activity (52–55), or preexisting DENV immunity in human cohorts (56) have led to similar observations, suggesting that the degree of conservation of neutralizing epitopes among DENV strains of the same serotype may be lower than initially thought, and that antigenic variation within a DENV serotype may limit the scope of protection of neutralizing antibodies induced by any single vaccine candidate.

We showed here that EDIII-specific mAbs 3H5-1 and MA1-27093 were markedly more potent in neutralizing DENV2 strain 16681 than D2Y98P-PP1. However, EDIII sequences of both DENV2 strains are highly conserved, with a single mutation at position 390. Interestingly, Sukupolvi-Petty et al. (34) reported that an N→Y mutation at this position reduced binding of 3H5-1 by 40%; similarly, the N→S mutation between 16681 and D2Y98P-PP1 may affect viral neutralization by EDIII-specific mAb. However, it is unlikely that this mutation alone is responsible for the 10-fold or greater difference in PRNT₅₀ values observed, given that the same residue is reportedly not critical for the binding of DENV2 type-specific or DENV complex-reactive antibodies (37). Rather, as previously proposed (52), it is plausible that amino acid differences in other domains of the E protein may lead to differential conformation of EDIII, thereby affecting antibody binding and neutralizing activity. Consistent with this hypothesis, it has been recently reported that mutation of a single residue in the West Nile virus E protein I-II hinge domain impacts virus “breathing” and resulted in an approximately 70-fold change in neutralization efficacy by a mAb targeting a cryptic epitope (57). Additionally, it is important to note that unlike the murine antibody response to DENV, EDIII-reactive antibodies represent only a minor fraction of the human antibody repertoire (33, 58, 59) — consequently, the above observations may be limited to the mouse model. Nevertheless, human EDIII-specific antibodies are potently neutralizing (59) and may still contribute significantly to protection (60). Whether human PDK53 immune serum exhibits similar variability in neutralizing activity against different strains of DENV2 remains to be investigated.

In an attempt to understand the potential role and impact of maternal vaccine-induced antibodies on vaccination efficacy, we investigated the outcome of PDK53 vaccination in pups born to PDK53-immunized dams. Our study did not find evidence of seroconversion and instead supported the occurrence of maternal antibody interference, a phenomenon that is well documented in human and veterinary medicine (29). Our observations are also consistent with an earlier study reporting the ability of maternal DENV2 antibodies to block seroconversion despite 2 injections of DENV2 New Guinea C (NGC) strain in BALB/c pups (61). Several mechanisms have been proposed to explain maternal antibody interference that may not be mutually exclusive. Maternal antibodies may bind critical viral epitopes, thereby preventing access by naive B cells; maternal antibodies may also neutralize the DENV vaccine, thus preventing viral replication and, in turn, impairing the immune response; finally, the third mechanism involves the formation of immune complexes between maternal antibodies and dengue virions that crosslink B cell receptor and inhibitory FcγRIIB, thereby leading to a negative intracellular signaling event that blocks B cell activation (29). Overcoming this inhibition requires signaling through CD21 and type I IFN receptor (62). Of note, the absence of type I IFN receptor in the A129 mouse strain may imply a predisposition to maternal antibody interference in these mice.

Despite their impact on B cells, maternal antibodies do not inhibit the T cell response (29) and our data indicate that CD8⁺ T cells are indeed robustly activated following administration of PDK53 vaccine to pups born to PDK53-immunized dams. It has been determined that murine and human CD8⁺ T cells preferentially target DENV nonstructural proteins (17, 38), thus making viral replication an important requirement for CD8⁺ T cell activation. We have shown that CD8⁺ T cells from vaccinated pups born to PDK53-immunized dams responded to NS4B_{99–107} peptide stimulation with vigorous IFN-γ production, which strongly suggests that viral replication had occurred despite high neutralizing levels of maternal antibodies. Such a phenomenon has been previously described or alluded to by other groups. Simmons et al. (63) sampled Vietnamese infants born to DENV-immune mothers and found a substantial proportion that developed dengue disease despite high levels of maternal neutralizing antibodies. BALB/c mice passively transferred with highly neutralizing antibodies against tick-borne encephalitis virus (TBEV) developed

high levels of anti-NS1 antibodies upon TBEV infection, supporting the notion that viral replication had occurred despite neutralizing antibodies (64). Watanabe et al. (65) showed that AG129 mice inoculated with DENV-antibody immune complexes, which were greater than 98% neutralized *in vitro*, still developed viremia. The ability of viruses to replicate despite the presence of neutralizing antibodies may be partially explained by the diversity of the antibody repertoire following DENV infection, which consists mainly of cross-reactive, poorly neutralizing antibodies with only a small fraction displaying serotype-specific, highly neutralizing properties (52, 53, 58, 59, 66, 67). A substantial proportion targets the prM protein and these antibodies possess little neutralizing but significant enhancing activities (66, 68). The presence of different antibody species with distinct neutralizing and enhancing profiles implies a scenario where a certain proportion of virions may escape neutralization and enter susceptible host cells via ADE. This mechanism may explain how PDK53 can still replicate and produce nonstructural proteins to activate T cells in pups born to PDK53-immune dams.

Our data strongly support a key role for CD8⁺ T cells in protection in the presence of enhancing, interfering maternal antibodies. Consistently, viruses often elicit substantial activation and expansion of CD8⁺ T cells in humans (69, 70) and mice (71, 72). Subsequently, CD8⁺ T cells mediate viral clearance by killing infected cells through perforin release and Fas/FasL interaction, and indirectly through secretion of IFN- γ , TNF- α , and chemokines (73). Historically, cross-reactive memory CD8⁺ T cells were thought to contribute to the immunopathogenesis of severe disease during a secondary heterologous DENV infection, according to the OAS hypothesis (2, 4). Recent experimental evidence, however, supports a protective role for DENV-specific CD8⁺ T cells. In mice, CD8⁺ T cell depletion prior to DENV infection resulted in elevated viral loads, whereas immunization with immunodominant CD8⁺-specific DENV peptides led to reduced viral loads after homologous challenge (17). Also, adoptive transfer of DENV-primed CD8⁺ T cells protected recipient mice from ADE (20). Finally, a study on the roles of humoral and cellular immune responses induced by a DENV replicon vaccine showed that, depending on the circumstances, vaccine-induced humoral responses could either protect mice or exacerbate dengue disease, whereas CD8⁺, but not CD4⁺, T cells were clearly protective (74).

Furthermore, multifunctionality of T cells (i.e., the ability to perform 2 or more functions, including degranulation and production of cytokines and chemokines) is an indicator of the quality of the T cell response and has been shown to correlate with disease nonprogression and protection in various models (43). In humans, a study conducted in the Sri Lankan hyperendemic area revealed an association between vigorous response by multifunctional CD8⁺ T cells and protection from DENV disease (19). In a Thai cohort study, higher frequencies of DENV-specific TNF- α -, IFN- γ -, and IL-2-producing T cells were associated with reduced disease severity during a secondary infection (75). Importantly, in the context of vaccine development, the ability of a DENV vaccine to induce multifunctional T cells has become an important criterion to evaluate vaccine efficacy (23–26). Consistently, we observed a high frequency of bifunctional (CD107a⁺IFN- γ ⁺) CD8⁺ T cells in PDK53-immunized mice after D2Y98P-PP1 challenge that may be instrumental for efficient viral clearance and superior protection.

We demonstrated DENV-specific *in vivo* cytotoxic activity in PDK53-vaccinated mice that correlated with high frequency (~30%) of CD8⁺ T cells expressing CD107a at their surface. This finding suggests that CD8⁺ cytotoxic T lymphocyte-mediated (CTL-mediated) killing of infected cells is an important, if not the main, mechanism of protection. Interestingly, although at low level (~0.8%), CD107a expression was also detected in CD4⁺ T cells. DENV-specific CD4⁺ CTLs were previously described and shown to lyse target cells (18, 21, 76) but the low frequency of such cells in the current study suggests a minor role in protection. A large fraction (~19%) of CD8⁺ T cells in the vaccinated–challenged group also expressed IFN- γ . Although IFN- γ can potentially augment ADE by upregulating the expression of Fc γ receptor (77), it raises the antiviral state of the host cell and enhances the activation of DENV-infected dendritic cells, thereby contributing to rapid virus clearance (78). Interestingly, TNF- α -expressing cells were rare even within the vaccinated–challenged group, suggesting that this cytokine is dispensable for protection against homotypic infection. In fact, cross-reactive memory T cells exhibiting increased TNF- α production were implicated in the immunopathogenesis of severe dengue disease (3). Thus, limited production of TNF- α by vaccine-primed T cells may be a desirable feature of the vaccine candidate.

The contribution of CD4⁺ T cells, in contrast, appears minimal, as evidenced by the mild increase in effector cells following vaccination or infection, the inability of adoptively transferred cells to protect recipients from lethal challenge, and the low frequency of cells that responded to *ex vivo* antigenic

stimulation. Our observations are consistent with other studies reporting that CD4⁺ T cells are not required for the primary B or CD8⁺ T cell response to DENV (18), and that memory CD8⁺ T cells do not require CD4⁺ T cells to become activated (79).

The current study has only examined the vaccine efficacy in the context of a single DENV serotype, whereby the monovalent vaccine candidate and the challenge DENV strain are from the same serotype. As DENV CD8⁺ T cell epitopes are relatively conserved since they are mainly found within nonstructural proteins for both mouse and human (17, 19), they are expected to confer some level of cross-protection among the 4 DENV serotypes. Consistently, cross-protective multifunctional CD8⁺ T cells were detected in human volunteers immunized with the DENVax tetravalent vaccine candidate (25). In mice, DENV4-induced CD8⁺ T cells were found to mediate short-term protection against heterotypic DENV2 reinfection (22). Furthermore, HLA*B0702 transgenic mice immunized with relevant human CD8⁺ T cell epitopes displayed enhanced viral clearance upon heterologous DENV infection (80). Thus, it is not unreasonable to think that a subset of PDK53-primed CD8⁺ T cells may be able to confer protection against DENV1, -3, and -4. Since the DENVax tetravalent candidate consists of a PDK53 backbone where the prM- and E-encoding genes have been swapped with those from the 3 other serotypes, a potential CD8⁺ T cell-mediated protection will have to rely on cross-reactive cells upon heterotypic infection. However, the diversity of human HLA alleles may lead to considerable variations in the magnitude of the protective efficacy conferred by these cross-reactive T cells. In the context of vaccination of infants with a tetravalent formulation where maternal interference is still likely to occur since most of the DENV-specific antibodies are cross-reactive and nonneutralizing, generation of a broad cross-protective CD8⁺ T cell-mediated protection will be critical.

In conclusion, we have demonstrated the ability of the PDK53 vaccine candidate to confer protection in the presence of enhancing, interfering maternal antibodies. Our work further supports a major protective role for vaccine-induced CD8⁺ T cells and advocates for the development of vaccine candidates that can induce a vigorous CD8⁺ T cell response. Assuming that they meet the safety criteria, such vaccine candidates will be extremely relevant and useful to protect babies born to vaccinated mothers in highly endemic areas where waning levels of maternal antibodies is a risk factor for DHF/DSS during a primary DENV infection (5).

Methods

Cell lines, viruses, and antibodies. BHK-21 (baby hamster kidney) and C6/36 (*Aedes albopictus* larva) cell lines were obtained from the American Type Culture Collection (ATCC). Cell culture media and reagents were purchased from Gibco, Thermo Fisher Scientific. Fetal bovine serum (FBS) was heat inactivated (56°C, 30 minutes) before use. BHK-21 cells were routinely maintained in RPMI 1640 medium supplemented with 10% v/v FBS and incubated at 37°C, 5% CO₂. C6/36 cells were maintained in Leibovitz's L-15 medium supplemented with 10% v/v FBS and incubated at 28°C without CO₂.

DENV2 strains 16681 (GenBank accession KU725663) and PDK53 (accession KU725664) were obtained from EE Ooi (Duke-NUS Medical School). DENV2 strain D2Y98P-PP1 (accession JF327392) was plaque purified from D2Y98P, a 1998 Singapore human isolate (31). Viruses were passaged no more than 3 times in C6/36 cells; infected cell culture supernatant was centrifuged at 12,000 g, 4°C for 30 minutes to pellet cell debris and stored at -80°C until further use. Viral titers were determined by plaque assay in BHK-21 cells (see below).

3H5-1 mAb was a gift from En Wei Teo (Department of Microbiology and Immunology, National University of Singapore). This is an affinity-purified, DENV2 type-specific murine IgG1 mAb. Antibody concentration was determined by the bicinchoninic acid (BCA) assay (Thermo Fisher Scientific). MA1-27093 was purchased from Thermo Fisher Scientific. This is an affinity-purified, DENV complex-specific murine IgG2a mAb.

Plaque assay. BHK-21 cells were seeded in 24-well tissue culture plates (Nunc, Thermo Fisher Scientific) 1 day earlier at a density of 50,000 cells per well. Virus suspension was serially diluted in RPMI 1640 supplemented with 2% v/v FBS. One hundred microliters of each dilution was added to BHK-21 monolayers and incubated for 1 hour at 37°C, 5% CO₂. Monolayers were eventually overlaid with RPMI 1640 medium supplemented with 2% v/v FBS and 1% w/v carboxymethylcellulose (Sigma-Aldrich) and further incubated for 4, 6, and 7 days, depending on the virus strain (D2Y98P-PP1, 16681, and PDK53, respectively). Plaques were visualized by pouring off the overlay medium and staining monolayers with a solution of 0.2% w/v crystal violet and 4% v/v paraformaldehyde. Plaques were manually counted for the determination of viral titers. Triplicates of each dilution were performed.

Plaque reduction neutralization test (PRNT). A variation of the Russell and Nisalak PRNT (81) was performed. Viruses were adjusted in RPMI 1640 supplemented with 2% v/v FBS to approximately 30 PFU per 100 μ l. Two-fold serial dilutions of purified mAb or heat-inactivated (56°C, 30 minutes) immune sera were prepared with diluted virus. The antibody-virus mixtures were incubated at 37°C for 1 hour before 100 μ l was added to BHK-21 monolayers. Plaque assay was performed as described above. Each plate contained virus-only reference wells from which plaque reduction was calculated. Data were plotted in GraphPad Prism (version 5.02) and the PRNT₅₀ titer was determined by nonlinear regression. Lower limit of detection was set at a serum dilution of 1:20; PRNT₅₀ titer below the limit of detection was assigned a nominal value of 10.

Indirect ELISA. Recombinant DENV2 NS1 protein (82) was a gift from Subhash G. Vasudevan (Duke-NUS Medical School). DENV2 particles were semipurified by polyethylene glycol 8000 (PEG; Sigma-Aldrich) precipitation for use as coating antigens. Briefly, D2Y98P-PP1- or 16681-infected C6/36 cell culture supernatant was clarified by centrifugation before an equal volume of 2 \times PEG solution (14% w/v PEG, 4.6% w/v NaCl) was added. The suspension was stirred overnight at 4°C and precipitated virions were pelleted by centrifuging at 12,000 g for 30 minutes at 4°C. Pellets were thoroughly dried before resuspending in sterile PBS. Protein concentration was determined using the BCA assay. Virions were UV inactivated (500 mJ/cm²) before use. Ninety-six-well high binding microplates (Corning) were coated with 100 ng/well of DENV2 or 10 ng/well of NS1 protein in PBS overnight at 4°C. Plates were washed with PBS supplemented with 0.1% v/v Tween-20 and blocked with 2% w/v BSA for 1 hour at 37°C. Four-fold serial dilutions of serum samples were added to the plates and incubated for 1 hour at 37°C. HRP-conjugated goat anti-mouse IgG (H+L) (Bio-Rad) was applied at 1:3,000 dilution. Plates were subsequently developed with SIGMAFAST OPD (Sigma-Aldrich) for 30 minutes at room temperature. Reaction was stopped with 1 M H₂SO₄ and absorbance was read at 490 nm using the Bio-Rad model 680 microplate reader. Endpoint titer was defined as the reciprocal of the serum dilution producing a reading twice that of background. Data were plotted in GraphPad Prism and endpoint titer was determined by nonlinear regression. The lower limit of detection was set at a serum dilution of 1:50; antibody titer below the limit of detection was assigned a nominal value of 12.5.

Mice. Sv/129 mice deficient in type I IFN receptors (A129) were purchased from B&K Universal and housed under murine pathogen-free conditions in individual ventilated cages. Pups were weaned at 3 weeks of age and both males and females were used in the study.

Immunization and challenge experiments. DENV viruses were administered via the intravenous (i.v.) route under anesthesia using a cocktail of ketamine (150 mg/kg), diazepam (2.25 mg/kg), and atropine (0.5 mg/kg) prior to the procedure. Five-wk-old female A129 were administered once with 3×10^5 PFU of PDK53 vaccine. Blood was collected at 8 weeks of age via retro-orbital puncture to assess PRNT₅₀ titers before breeding was initiated at 9 weeks of age with a dengue-naive A129 male. Pups were immunized with 10^5 PFU of PDK53 at 3 weeks of age. For challenge experiments, pups were infected with 16681 or D2Y98P-PP1 virus at 3 or 6 weeks of age. Clinical symptoms were scored as follows: 0, no observable symptoms; 1, ruffled fur; 2, diarrhea; 3, hunching; 4, severe hunching, both eyes shut, lethargy. Endpoint of the study was set at a clinical score of 4 and mice were euthanized by CO₂ asphyxiation.

Quantification of viral loads. For assessment of viremia, blood was collected under anesthesia by retro-orbital puncture. After centrifugation (2,000 g, 4°C, 10 minutes), sera were collected and stored at -80°C until further analysis. For organ collection, mice were euthanized by CO₂ asphyxiation and perfused with 40 ml of sterile PBS. Organs were harvested under sterile conditions and snap-frozen in liquid nitrogen. The jejunum was cleared of luminal contents prior to snap-freeze. All organs were eventually stored at -80°C until further analysis. Organs were weighed and homogenized in 2 ml of RPMI 1640 supplemented with 2% v/v FBS. Cell debris was pelleted by centrifugation and the supernatant was filter sterilized (0.22 μ m) and assayed. Sera were diluted in RPMI supplemented with 2% v/v FBS and filter sterilized. Levels of infectious virions were determined by plaque assay. Viremia was expressed as PFU per ml of serum; organ viral load was expressed as PFU per gram of wet weight.

Isolation of PBMCs and splenocytes for flow cytometry. For isolation of PBMCs, approximately 200 μ l of blood was collected from each mouse in an Eppendorf tube containing 50 μ l of 0.5 M EDTA (First BASE Laboratories Sdn Bhd) and thoroughly mixed. Blood was then treated with cell lysis buffer (2.075% w/v NH₄Cl, 0.25% w/v NaHCO₃, 0.25 mM EDTA) for 3 minutes at room temperature. Lysis buffer was quenched and cells were washed with MACS buffer (sterile PBS supplemented with 1 mM EDTA and 2% v/v FBS). For isolation of

splenocytes, organs were aseptically harvested and mashed through 70- μ m sterile cell strainers (SPL Life Sciences) to generate single-cell suspensions. Red blood cells were lysed by lysis buffer. Viable cell counts were manually determined using a hemocytometer and trypan blue exclusion.

Flow cytometry. All antibodies and reagents were purchased from eBioscience with the exception of Pacific Blue-anti-mouse CD4 (BD Biosciences, clone RM4-5), Alexa Fluor 700-anti-TNF (BD Biosciences, clone MP6-XT22), and PE-Cy7-anti-CD107a (BioLegend, clone 1D4B). DENV2 peptides NS3₁₉₈₋₂₁₂ (GKTKRYLPAIVREAI) and NS4B₉₉₋₁₀₇ (YSQVNPITL) were synthesized by Mimotopes Pty Ltd. PBMCs (3×10^5 /well) or splenocytes (1×10^6 /well) were added to a 96-well U-bottom plate (SPL Life Sciences) for surface staining; a second plate was prepared for peptide stimulation and intracellular staining (ICS). For assessment of T cell proportions, cells were labeled with fixable viability dye eFluor 780, APC-anti-CD3e (clone 145-2C11), Pacific Blue-anti-CD4 (clone RM4-5), PerCP-Cy5.5-anti-CD8a (clone 53-6.7), PE-anti-CD44 (clone IM7), and FITC-anti-CD62L (clone MEL-14). For peptide stimulation and ICS, cells were resuspended in 200 μ l complete T cell medium (RPMI 1640 supplemented with 10% v/v FBS, 50 μ M β -mercaptoethanol, 2 mM L-glutamine, 10 mM HEPES, and 100 U/ml Pen/Strep) and incubated with 3 μ g/ml NS3₁₉₈₋₂₁₂ and 0.1 μ g/ml NS4B₉₉₋₁₀₇ peptide in the presence of brefeldin A and monensin for 5 hours. Phorbol 12,13-dibutyrate (PdBu) and ionomycin treatment was used as a positive control. To detect expression of CD107a, PE-Cy7-anti-CD107a (clone 1D4B) was added at the start of incubation. Cells were washed with MACS buffer and labeled with fixable viability dye eFluor 780, APC-anti-CD3 (clone 145-2C11), Pacific-Blue-anti-CD4 (clone RM4-5), and PerCP-Cy5.5-anti-CD8a (clone 53-6.7) before treating with Foxp3 Transcription Factor Fixation/Permeabilization buffer (eBioscience). Intracellular cytokines were detected using Alexa Fluor 488-anti-IFN- γ (clone XMG1.2), Alexa Fluor 700-anti-TNF (clone MP6-XT22), and PE-anti-IL-5 (clone TRFK5). Single color compensation controls were prepared using UltraComp eBeads; live/dead control was prepared by mixing equal numbers of live and heat-killed (95°C, 30 seconds) cells. Samples were read on an LSRFortessa (BD Biosciences) and analyzed using FlowJo software. Boolean gating was applied to determine all possible combinations of T cells that expressed effector functions.

Isolation of T cells for adoptive transfer. A129 mice were immunized with 10^5 PFU of PDK53 at 5–7 weeks of age. Seven days later, the mice were euthanized and their spleens and lymph nodes (brachial, axillary, and popliteal) were harvested. Nonimmunized mice served as a source of naive T cells. Single-cell suspensions were prepared by mashing organs through sterile 70- μ m cell strainers. Lymphocytes were isolated using Ficoll-Paque PLUS (GE Healthcare). CD4⁺ and CD8⁺ T cells were enriched using CD4 (L3T4) and CD8a (Ly-2) MicroBeads (Miltenyi Biotec), respectively, according to the manufacturer's instructions, and resuspended in sterile PBS. Purity of T cells (89.6%–93.4%; data not shown) was determined by flow cytometry.

In vitro activation of naive CD8⁺ T cells. CD8⁺ T cells isolated from 5- to 7-wko naive A129 mice were activated by incubating them with anti-CD3 and anti-CD28 antibodies. Briefly, wells of a 48-well tissue culture plate were coated with 1 μ g/ml of anti-mouse CD3 (eBioscience, clone 145-2C11). CD8⁺ T cells purified by MicroBeads were plated at a concentration of 3×10^6 cells/ml, and anti-mouse CD28 (eBioscience, clone 37.51) was added at a concentration of 1 μ g/ml to the culture. After 3 days incubation, activated CD8⁺ T cells were harvested for adoptive transfer experiments.

Adoptive transfer and lethal challenge. CD4⁺ or CD8⁺ T cells (1.5×10^7 or 6×10^6 as indicated) were adoptively transferred to 5- to 7-wko mice born to PDK53-immunized dams via the i.v. route under anesthesia. One day later, 10^7 or 10^6 PFU of D2Y98P-PP1 virus (as indicated) were i.v. administered under anesthesia and mice were monitored daily for symptoms.

In vivo cytotoxicity assay. The assay was performed according to a previously described protocol (83). Briefly, splenocytes were harvested from naive mice and pulsed with 1 μ g/ml NS4B₉₉₋₁₀₇ or OVA SIINFEKL peptide. Cells were washed and then labeled with 0.5 or 5 μ M CFSE, respectively, to enable identification of the 2 populations by flow cytometry. NS4B₉₉₋₁₀₇-pulsed (1×10^7) and SIINFEKL-pulsed (1×10^7) splenocytes were mixed in equal proportion and injected i.v. into 6-wko A129 mice born to PDK53-immunized dams and immunized, or not, with PDK53 at 3 weeks of age. After 4 hours, mice were euthanized and spleens were harvested for the analysis of CFSE-labeled cell populations. The mixture of pulsed splenocytes before transfer was also analyzed by FACS (plate control). The ratio of the proportions of SIINFEKL-pulsed to NS4B₉₉₋₁₀₇-pulsed cells was calculated to derive percentage killing: $(1 - [\text{plate control ratio/experimental ratio}]) \times 100$. Negative values were assigned "0" arbitrarily.

Statistics. Nonparametric analyses were done using GraphPad Prism software. Two-sided Mann-Whitney test was performed to compare differences between 2 samples; Kruskal-Wallis test was performed for multiple comparison. For matched samples, 2-sided Wilcoxon signed-rank test was performed. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; not significant, $P > 0.05$.

Study approval. All the animal experiments were carried out in accordance with the guidelines of the National Advisory Committee for laboratory Animal Research (NACLAR). Animal facilities are licensed by the regulatory body Agri-Food and Veterinary Authority of Singapore (AVA). The described animal experiments were approved under the protocol number R14-0097 by the Institutional Animal Care and Use Committee (IACUC) from National University of Singapore (NUS) located at CeLS building, 28 Medical Drive, Singapore 117456.

Author contributions

JHL and SA designed the experiments and wrote the manuscript. JHL, YLC, PXL and JMMG, performed the experiments. EEO provided materials and intellectual input.

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