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PROTECTIVE IMMUNE RESPONSE TO DENGUE VIRUS INFECTION AND VACCINES: PERSPECTIVES FROM THE FIELD TO THE BENCH

Topic Editors
Simona Zompi and Scott Halstead

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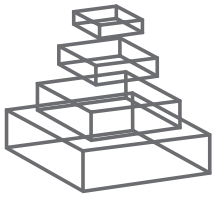

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PROTECTIVE IMMUNE RESPONSE TO DENGUE VIRUS INFECTION AND VACCINES: PERSPECTIVES FROM THE FIELD TO THE BENCH

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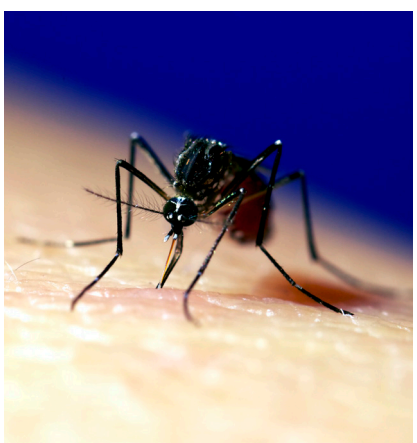

Aedes aegypti in the process of feeding on a dengue infected patient. Copyright owned by Oxford University Clinical Research Unit.

Dengue is the most important mosquito-transmitted viral disease in humans. Half of the world population is at risk of infection, mostly in tropical and sub-tropical areas. The World Health Organization (WHO) estimates that 50 to 100 million infections occur yearly, with 50,000 to 100,000 deaths related to dengue, mainly in children. Recent estimates show higher numbers, up to three times more, with 390 million estimated dengue infections per year, among which 96 million apparent infections (Bhatt et al. 2013). Initially localized to South-East Asia, dengue virus (DENV) started its spread in Latin America in the 80's. Little is known about DENV spread in Africa, but multiple seroprevalence surveys over several years are now clearly showing endemic areas in East and West Africa (Brady et al. 2013). Finally, due to global warming and intense traveling there is a risk of global spread towards more temperate regions, and both US Key islands (FL) and southern Europe recently faced DENV outbreaks.

There are currently no specific treatments or vaccines available. Even though several dengue vaccines are in the pipeline, clear correlates of protection are still lacking. The recent failure of the live-attenuated Sanofi vaccine Phase 2b trial (Sabchareon et al. 2013) and the lack of correlation

between clinical protection and *in vitro* neutralization assays, clearly underlines the necessity to better understand the role of the different components of the immune system in protection against dengue virus infection and the requirement for the development of additional and/or improved predictive assays. The aim of this research topic is to provide novel data, opinions and literature reviews on the best immune correlates of protection and recent advances in the immune response to DENV infection that can allow rapid progress of dengue vaccines. Authors can choose to submit original research papers, reviews or opinions on pre-clinical or clinical observations that will help unify the field, with perspectives from epidemiology, virology, immunology and vaccine developers.

This research topic will discuss different aspects of the protective immune response to DENV that can influence vaccine development. It will include a review of epidemiological data generated in the field, which will address spatio-temporal diversity of DENV epidemics, the importance of cross-reactive protection and of the time-interval between infections as a predictor of disease. It will further include a review of the role of both the innate and adaptive immunity in DENV infection control, and discuss the usefulness of new improved animal models in dissecting the role of each immunological compartment, which will help define new correlate of immune protection. New data concerning the DENV structure and anti-dengue antibody structure will address the necessity of improved neutralization assays. The ultimate test to prove vaccine efficacy and study immune correlates of protection in humans before large trials will open up the discussion on human DENV challenges using controlled attenuated viral strains. Finally, the role of vaccines, administered in flavi-immune populations, in the modification of future epidemics will also be approached and will include novel studies on mosquitoes infection thresholds.

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Protective immune responses to dengue virus infection and vaccines: perspectives from the field to the bench

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Keywords: dengue, antibodies, T cells, myeloid cells, NK cells, immunity, protection, vaccines

Dengue research is in turmoil following confusing efficacy reports from large-scale phase III clinical studies on the lead candidate tetravalent dengue vaccine (1–3). Within the context of the current understanding of immunity in dengue or immunity to other vaccines, the observed failures of protection cannot be adequately explained. These results comprise the background to this review of contemporary research on protective immunity in dengue, summarized by Slifka (4). Studies on wild-type dengue virus (DENV) infections of humans since World War II have revealed a consistent pattern of cross-protection after a single DENV infection against infection with a different DENV. Inapparent infections or mild disease accompany sequential DENV infections spaced at relatively short intervals (<1.4–1.9 years), while overt and severe disease accompany sequential infections at longer intervals. Grange et al. provide an analytical review of inapparent DENV infections published so far in the literature (5). It has been asked whether these inapparent infections serve as a major reservoir for the sustained infection of *Aedes aegypti*. Parameters of infection of *A. aegypti* by feeding on humans with dengue illnesses described here by Carrington et al. are a model for research directed at answering this question (6). An overview report by Endy (7) on the spectrum of human responses to wild-type DENV infection, from inapparent to hospitalized severe dengue, provides evidence that heterotypic DENV plaque-reduction neutralizing antibodies do not predict protection against a second DENV. This was the central feature of the Sanofi tetravalent dengue vaccine trial in Thai children (1). DENV 2 neutralizing antibodies uniformly were raised by three doses of vaccine yet failed to protect against symptomatic DENV 2 infections (1).

A longitudinal study on human immune responses to wild-type DENV infection describes how heterotypic immunity modulates disease, including evidence that cellular immunity contributes to protection (8). Weiskopf and Sette show that CD8+ T cells contribute to protection against disease with second DENV infections by targeting epitopes on non-structural antigens (8). In the Sanofi tetravalent chimeric vaccine, this T cell contribution may be missing as DENV non-structural proteins are not present in the vaccine, replaced by those of yellow fever (1–3). Studies on humans and animal models, summarized by Petitdemange et al. (9), find that antibody-dependent cell-mediated cytotoxicity (ADCC) and natural killer (NK) cells contribute to controlling early-stage viral infections. Since most human DENV infections are silent, NK cell-mediated protection may dominate (9, 10). This possibility is illustrated by observations from Cuba and Vietnam, reviewed by Beltran and Lopez-Verges (10), where differential distribution of alleles of the MHC-Class I chain-related genes A or B (MICA or MICB) suggest that NK responses have been suppressed in those individuals who developed severe disease. Cells of the immune system, including dendritic cells (DCs), monocytes (Mo), and macrophages (Mφ) serve as hosts of DENV infection. Immature DCs express DC-SIGN, a universal receptor for DENV. Immature DCs evolve from blood Mo that have migrated into the skin. In a mouse model, Schmid et al. show that immature DCs are initial sites of infection and once infected become mature and migrate to regional lymph nodes (11). Mature DCs lose DC-SIGN but gain Fc receptors (FcRs) and can be infected efficiently by infectious immune complexes. Different FcRs on Mo and Mφ interact with specific isotypes of IgG. When infectious DENV immune complexes attach to Mo and Mφ FcγRIIA a signal is sent suppressing interferon (IFN) type I production leading to the enhanced virus production (11).

A broad range of subhuman primate species are readily infected with wild-type or attenuated DENV. But, monkeys do not respond to infection with a disease mimicking the dengue vascular permeability syndrome (DVPS). Nonetheless, immune responses and protection to challenge in monkeys are closely similar to those observed in humans. Sariol and White review the utility and limitations of this animal model (12). Monkeys inoculated with tetravalent Sanofi and Takeda live-attenuated chimeric vaccines revealed the same dominance of DENV 4 and DENV 2-driven immune responses and protection observed in humans, respectively. T cell immune responses are scarcely studied in monkeys. In contrast, in mouse models, as shown by Zellweger and Shresta (13), adoptive transfer of T cells demonstrate the important contribution of the T cell component to protection following a first or second DENV infection. Mice lacking receptors to type I IFN, however, do have a pathophysiological response closely similar to DVPS. In these mice, suboptimal doses of DENV2 result in mild illness. In the presence of enhancing concentrations of dengue antibodies, i.e., sub-neutralizing concentrations that induce antibody-dependent enhancement or ADE, the same dose of DENV induces lethal disease (13).

The review is completed by a description by Ambuel et al. (14) of the successful immunization of cynomolgous monkeys

using two doses of a DENV2 chimeric tetravalent vaccine given at day 0 (rapid immunization strategy or RIS), as compared to the traditional prime and boost given 2 months later. As evidence of solid protection, when challenged with DENV 2, animals were protected against viremia with no boost in DENV 2 neutralizing antibodies, showing that the RIS induced a sterilizing immunity (14). Another important feature of this trial was the demonstration of T cell immunity to DENV 2 nonstructural proteins with collateral cross-reactive T cell immunity to other DENV types (14). This original article shows that RIS could be very useful in endemic areas to increase compliance to vaccination schedules and reinforce the necessity to study, in more detail, protective dengue-induced T cell immunity.

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Vaccine-mediated immunity against dengue and the potential for long-term protection against disease

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It is estimated that over 2.5 billion people are at risk for contracting dengue, a virus responsible for 50–390 million infections in addition to thousands of hospitalizations and deaths each year. There are no licensed vaccines available to combat this pathogen but substantial efforts are underway to develop live-attenuated, inactivated, and subunit vaccines that will protect against each of the four serotypes of dengue. Unfortunately, the results of a recent Phase IIb efficacy trial involving a tetravalent live-attenuated chimeric dengue virus vaccine have raised questions with regard to our current understanding of vaccine-mediated immunity to this important flavivirus. Here, we will briefly summarize these vaccination efforts and discuss the importance of informative *in vivo* models for determining vaccine efficacy and the need to establish a quantitative correlate of immunity in order to predict the duration of vaccine-induced antiviral protection.

Keywords: dengue, antibody, CD8$^+$ T cells, CD4$^+$ T cells, immunological memory, correlates of immunity, vaccines

INTRODUCTION

Dengue virus (DENV) represents a serious threat to the global community with transmission occurring in over 100 countries (1, 2). Within DENV, there are four distinct serotypes (DENV1, DENV2, DENV3, and DENV4) and each serotype has been found to cause human disease and mortality. DENV infection may result in a spectrum of disease, ranging from acute, debilitating febrile illness [dengue fever (DF)] to severe, life-threatening hemorrhagic disease [dengue hemorrhagic fever; DHF/dengue shock syndrome (DSS)]. Previous estimates indicated that there were 50–100 million cases of DENV infection and 250,000–500,000 cases of DHF/DSS each year, placing over 2.5 billion people at risk (1, 3–5). More recent analysis indicates that the overall burden of DENV could be as high as 390 million infections per year with as many as 96 million demonstrating clinically apparent disease (2). Vector control is currently the only means to reduce the risk of DENV transmission but development of a safe and effective vaccine is urgently needed in order to substantially reduce DENV disease worldwide.

FAILURE OF CYD-TDV VACCINE PHASE 2B TRIAL

A number of DENV vaccines are in various stages of development with several candidates undergoing testing in early-stage clinical trials (6–12). Of these candidates, the most advanced vaccine is ChimeriVax™, a recombinant flavivirus vaccine technology in which the envelope and PrM proteins of the attenuated vaccine strain of yellow fever 17D (YFV-17D) have been replaced with the proteins of each serotype of DENV. A tetravalent formulation containing four chimeric yellow fever vaccine strains of DENV, termed, CYD-TDV, has been developed and the results of the Phase IIb efficacy trial involving 4002 subjects have been reported (13). Since YFV-17D is a highly immunogenic and successful vaccine, it was generally anticipated that the recombinant CYD-TDV vaccine, would induce bona fide flavivirus-specific immune responses that would lead to strong and durable protective immunity against DENV infection. Unfortunately, overall vaccine efficacy was only 30% and differed by serotype. The majority of DENV infections were of the DENV2 serotype (48 cases in the per-protocol analysis), and it showed the lowest efficacy (9%). The highest vaccine efficacy was observed with DENV4 (100% efficacy based on 4 cases), followed by DENV3 (75% efficacy based on 3 cases), and DENV1 (56% efficacy based on 19 cases). The numbers of DENV cases in this study were relatively small and more data are needed to verify these potential rates of serotype-specific vaccine-mediated protection. Ongoing Phase III trials involving 30,000 volunteers have been initiated (NCT01373281 and NCT01374516) and these should provide more definitive data on vaccine efficacy across the four DENV serotypes.

The lack of observed efficacy in the CYD-TDV vaccine trial was unexpected since seroprevalence rates were high at baseline (e.g., ~70% seropositive to at least one of the four different DENV serotypes) and seroconversion to DENV2 reached 87% in the vaccinated group within 28 days after the first vaccination and 99% after the second vaccination (13). The conundrum of high vaccine immunogenicity but low protective efficacy has led to many questions regarding the lack of protection and the potential factors that may be involved. Vaccine interference from pre-existing anti-flavivirus immunity within the DENV-endemic population is one possibility or maybe the neutralizing assays used to measure immunogenicity are not able to distinguish between protective and non-protective levels of immunity. Alternatively, T cell-mediated immunity may be important for DENV-specific protection and antiviral T cell responses were not measured in this study. Overcoming pre-existing immunity is known to be a

problem for the live-attenuated DENV vaccines and even booster vaccinations must be separated by long intervals (e.g., 0, 6, 12-month vaccination schedule) (14, 15) or the "boosting" effect of secondary or tertiary vaccination is dampened by the immunity generated by the prior vaccinations. Vaccine interference is a common problem among all of the tetravalent live-attenuated DENV vaccine formulations, resulting in biased neutralizing antibody responses to some, but not all DENV serotypes until multiple vaccinations have been performed (16). Within an endemic community, it is possible that one or more of the vaccine strains of DENV are inhibited from efficient replication in the host and result in reduced induction of homotypic neutralizing antibodies to a broader number of DENV serotypes. Moreover, the cohorts at the site of the CYD-TDV trial also had high levels of pre-existing immunity to other flaviviruses (e.g., 78–80% seropositive for JEV) (13) and it is unclear if immunity to multiple flaviviruses is having a positive or negative impact on vaccine efficacy in the field. In addition to live, attenuated DENV vaccines such as CYD-TDV, there are several non-replicating vaccine approaches currently in clinical development including formalin-inactivated whole virion vaccines (NCT01502735, NCT01666652, and NCT01702857) and DENV envelope subunit protein vaccines (NCT00936429 and NCT01477580) and it will be interesting to learn if these non-replicating vaccine approaches suffer the same challenges as the live, attenuated DENV vaccines or if they are able to overcome viral interference in DENV-endemic communities.

Dengue virus-specific neutralizing assays are a key measurement of vaccine-induced antiviral immunity but there is considerable debate over the best approach to performing these assays. The 50% plaque-reduction neutralizing test ($PRNT_{50}$) assay used to measure antiviral immunity in the CYD-TDV Phase IIb trial has come into question since it was performed in Vero cells (16) and some have proposed that DENV strains should be grown in other cell types or that primary DENV strains isolated directly from acutely infected DENV patients be used in the analysis (17). It may not be feasible to perform standardized neutralization assays under GLP compliance using direct DENV isolates from human serum but these questions nevertheless further illustrate the point that despite the publication of a WHO guidance document (18), there is still little consensus among the scientific community on how neutralizing titers to DENV should be performed or which DENV strains should be used in this crucial analysis of antiviral immunity. Although the Phase IIb trial followed published WHO guidelines on performing DENV neutralization assays (18), these immunological assays were not performed with reference strains of each DENV serotype, but were instead performed with the individual chimeric CYD-TDV vaccine strains of virus that represented each DENV serotype (13). This could be problematic in the interpretation of seroconversion as well as in determining the true magnitude of DENV-specific antibody responses. For example, vaccine-induced neutralizing antibodies to the homologous CYD-TDV vaccine virus strains are typically 2-fold to >10-fold higher then the results obtained when non-recombinant wild-type strains of DENV of the same serotype are used for determining neutralizing titers (19). These results are not unique to CYD-TDV since similar results have been observed with a chimeric YFV vector expressing JEV envelope proteins (20) and a chimeric DENV vector expressing WNV envelope proteins (21). In each case, immunization with the vaccine strain of recombinant virus elicited higher antibody responses to the vaccine strain of virus than to the wild-type target strain of flavivirus (22). This means that in terms of the CYD-TDV Phase IIb trial, the high serotype-specific seroconversion rates observed against CYD-TDV strains of recombinant virus may have been lower if reference strains of wild-type DENV had been used in the $PRNT_{50}$ assays.

ROLE OF VACCINE-INDUCED T CELLS IN PROTECTION AGAINST FLAVIVIRUS INFECTION

With DENV (23) or West Nile virus (24–26), $CD8^+$ T cells can protect mice against viral infection, but pre-existing T cell memory is not absolutely required for protection since passive transfer of immune serum or neutralizing monoclonal antibodies can also mediate protection against lethal challenge (27, 28). In the AG129 mouse model of DENV infection, antiviral antibodies appear to play a greater role than T cells in protection against DENV challenge (29) and administration of neutralizing monoclonal antibodies can provide full protective immunity (27). However, the role of human T cell-mediated immunity in protection or pathogenesis during flavivirus infection remains unclear. For instance, although higher antiviral T cell responses to DENV have been found during DHF (30), it is uncertain if the antiviral T cell response is involved with causing disease or if it is instead an epiphenomenon in response to the higher viral load associated with DHF. Higher antiviral T cell responses/IFNγ production have also been associated with lower disease during DENV infection (31, 32), although it is unknown if this is due to a direct relationship to antiviral T cell immunity or if it is possible that higher antiviral T cell responses represent a biomarker indicative of better antiviral antibody responses. Primary infection with YFV-17D (33–36) or DENV (30, 37) induces strong antiviral T cell responses that are mounted against all viral proteins, although most are directed toward non-structural proteins. Most clinical studies are limited to measuring associations and it is often difficult to determine a direct cause-and-effect relationship between T cell responses and viral burden because normal vaccine-mediated antiviral immune responses include induction of both humoral and cellular immunity. One way to directly determine if vaccine-induced human T cell responses play a role in antiviral immunity to flaviviruses is to immunize human subjects with a vaccine that elicits antiviral $CD4^+$ and $CD8^+$ T cell responses in the absence of a virus-specific neutralizing antibody response. Interestingly, these studies have been performed during the early clinical testing of the ChimeriVax™ vaccine platform (38, 39) (**Table 1**). ChimeriVax is constructed with eight YFV-17D non-structural proteins but the YFV-17D envelope and PrM proteins are replaced with the surface proteins from another flavivirus (8). In two studies, the envelope proteins and the associated neutralizing epitopes of YFV-17D were replaced with the envelope proteins (and their associated virus-specific neutralizing epitopes) of DENV2 (39) or JEV (38) and the role of pre-existing YFV-17D-specific T cells in antiviral immunity was determined (**Table 1**). When YFV-17D-naïve subjects were vaccinated/infected with YFV-17D (10^5 PFU/dose), 100% of the vaccinees became viremic. Likewise, when YFV-17D naïve individuals were infected with ChimeriVax vaccine strains

Table 1 | Analysis of viremia in human subjects with or without pre-existing antiviral T cell memory*.

	YFV-17D-Naïve				YFV-17D-Immune			
Challenge virus	**YFV-17D 10^5 PFU**	**YFV-DENV2 10^5 PFU**	**YFV-JEV 10^5 PFU**	**YFV-JEV 10^4 PFU**	**YFV-17D 10^5 PFU**	**YFV-DENV2 10^5 PFU**	**YFV-JEV 10^5 PFU**	**YFV-JEV 10^4 PFU**
n	5	14	6	6	6	14	6	6
Viremia (%)	100	57.1	83.3	83.3	0	78.6	83.3	100
AUC	56	20.7	21.7	48.3	0	50.4	58.3	50

**Two clinical studies were conducted to determine if pre-existing YFV-specific immunity would impact virus replication upon challenge with YFV-17D or chimeric versions of YFV-17D in which the envelope and PrM proteins of YFV-17D were replaced by the envelope and PrM proteins of either DENV2 (39) or JEV (38). The chimeric flaviviruses, YFV-DENV2 and YFV-JEV, have the same eight non-structural proteins as YFV-17D and contain the same $CD4^+$ and $CD8^+$ T cell epitopes, but can no longer be neutralized by YFV-17D-specific antibodies, thus providing the opportunity to measure T cell-mediated protection in the absence of neutralizing antibodies to the viral structural proteins; n, number of subjects per group; PFU, plaque forming units; AUC, area under the curve.*

expressing the DENV2 envelope/PrM proteins (YFV-DENV2; 10^5 PFU/dose) or JEV envelope/PrM proteins (YFV-JEV; 10^5 or 10^4 PFU/dose), viremia was observed in 57.1, 83.3, and 100% of subjects, respectively. This demonstrates that these chimeric viruses maintained viral fitness in their human host and readily induced viremia in flavivirus-naïve individuals with a measurable peak and duration of systemic infection. Vaccination of YFV-17D-immune subjects proved to be an insightful experiment because these individuals presumably have pre-existing antiviral $CD4^+$ and $CD8^+$ T cells to the yellow fever non-structural proteins encoded in the ChimeriVax vector, but would lack neutralizing antibody responses because the YFV-17D envelope and PrM genes in these recombinant viruses have been replaced by DENV2 or JEV envelope/PrM genes. Although YFV-17D (10^5 PFU/dose) failed to induce detectable viremia in YFV-17D-immune subjects (0% viremic), YFV-DENV2 (10^5 PFU/dose), and YFV-JEV (10^5 PFU/dose) caused viremia with infection rates that were similar to that observed in YFV-17D-naïve subjects. Even the lower dose of YFV-JEV (10^4 PFU/dose) infected 100% of the YFV-17D-immune subjects, indicating that antiviral T cell memory was insufficient for inhibiting low-dose viral challenge (**Table 1**). Together, these data suggest that pre-existing $CD8^+$ and $CD4^+$ memory T cell responses to 8/10 of the flavivirus proteins, including all of the non-structural proteins, failed to protect against flavivirus infection in an experimental model in which antiviral neutralizing antibody responses were absent.

Virus-specific T cells cannot block infection *per se* (this is best accomplished by neutralizing antibodies), but may be involved with reducing viral load once an infection has occurred. However, pre-existing T cell memory did not reduce the peak level of viremia or lower the duration of viremia by chimeric YFV-JEV or YFV-DENV2 (38, 39). This information was captured in the area under the curve (AUC) measurements that combined the magnitude and duration of viremia measurements and based on this assessment, pre-existing T cell memory in YFV-17D-immune subjects did not play a measurable role in reducing viral load after chimeric YFV-17D-based flavivirus infection (**Table 1**). In contrast, YFV-17D-immune subjects were fully protected against YFV-17D that express the homologous envelope proteins (38, 40). The protection in this case may be largely due to neutralizing antibodies since prior studies have demonstrated that adoptive transfer of immune serum alone provides partial to full protective immunity against lethal YFV in rhesus macaques (RM) (41), hamsters (42), and immunodeficient mice (43).

Although vaccine-induced T cell memory failed to prevent viremia, one would anticipate that another contribution of cellular immunity would be to modify disease upon flavivirus reinfection. However, amelioration of disease symptoms was not observed; following infection with YFV-DENV2, the incidence of myalgia, arthralgia, rash, and rigors was higher in YFV-17D-immune subjects compared to YFV-17D-naïve subjects (39) and following YFV-JEV infection, the only subject with a high fever (102.1°F) belonged to the YFV-17D-immune group. Of the other two cases of low-grade fever considered by the investigators to be possibly related to vaccination, these also occurred in the YFV-17D-immune group (38). It is important to keep in mind that these are relatively small clinical studies and it is possible that antiviral T cell memory plays a more substantial role in protection against wild-type flaviviruses or that they may function in a manner that was not measured in these clinical assessments. However, based on this work there appears to be little evidence that pre-existing vaccine-induced T cell memory is involved with prevention of secondary flavivirus infection, dissemination, or early disease progression. In addition, because YFV-17D-immune subjects would be expected to have pre-existing antibodies to as many as eight non-structural YFV proteins that are found in the recombinant YFV-JEV and YFV-DENV2 viruses, this work also suggests that non-neutralizing antibodies to these viral proteins are unlikely to play a major role in vaccine-mediated protection against flavivirus infection.

CONCERNS OF VACCINE-INDUCED ANTIBODY DEPENDENT ENHANCEMENT

The pathogenesis of DENV is complex and there has been considerable concern that vaccine-induced antibody dependent enhancement (ADE) of DENV infection could result in exacerbated disease among vaccinated individuals who have only partial immunity or low-level heterotypic immunity to secondary DENV infection (44–46). ADE is a phenomenon in which non-neutralizing antibodies or sub-neutralizing levels of virus-specific antibodies result in enhanced infection of Fc receptor-bearing cells (e.g., macrophages, monocytes) (44, 45, 47). Fortunately,

long-term monitoring of vaccinees in DENV-endemic countries has not revealed evidence of ADE. For example, one group found that 4/113 (3.5%) vaccine recipients had been hospitalized with DENV within 6.8 years after DENV vaccination whereas 14/226 (6.2%) unvaccinated, age-matched, and location-matched children were hospitalized due to DENV (45). Perhaps the most compelling evidence for a lack of vaccine-mediated ADE comes from the CYD-TDV Phase IIb trial (13). This study provided an example of measurable immunogenicity but low protective efficacy and would be expected to result in the highest likelihood of ADE. However, despite incomplete vaccine-mediated protection against the four serotypes of DENV (and a non-protective immune response to the circulating strain of DENV2), analysis of 2600 vaccinated children monitored for 2 years after vaccination (i.e., 5200 person-years) showed no increase in the rate or clinical severity of DENV infection among the vaccinated population. This is important safety information and provides further support for continued development of an effective DENV vaccine.

NEUTRALIZING ANTIBODIES AND THE DURATION OF VACCINE-MEDIATED IMMUNITY

It is often difficult to estimate how long protective vaccine-mediated immunity will last unless (a) the correlate of immunity has been established and (b) the levels of immunity are measured in longitudinal or cross-sectional studies for a prolonged period of time. For example, if neutralizing antibodies represent the correlate of immunity and the protective threshold is determined to be a $PRNT_{50}$ of 10, then measuring the magnitude and duration of $PRNT_{50}$ titers over time will provide valuable information on the durability of protective immunity (**Figure 1**). In some cases, vaccination will elicit low levels of immunity that are measurable, but reside below the threshold needed for protection (**Figure 1A**). An example of this would be the small subset of individuals who receive the MMR vaccine and develop anti-measles antibodies that are readily detected by ELISA, but are below the protective threshold of 0.2 IU/mL (48, 49). Alternatively, vaccines may elicit short-term protective immunity in which antibodies remain above the threshold of protection for a brief period of time before declining below the protective threshold (**Figure 1B**). There are several examples of vaccines that fall into this category including the acellular pertussis vaccine that provides 98% protection during the first year after completing the primary vaccination series, but then declines steadily to 71% protection by 5 years post-vaccination (50). Live, attenuated vaccines such as the pediatric varicella zoster vaccine also require two doses of virus because, although protective immunity is high shortly after vaccination, the levels of protection wane gradually after the first dose, resulting in significantly higher break-through cases of varicella within 5 years after primary vaccination (51). Longitudinal analysis of DENV1-specific neutralizing titers during CYD-TDV vaccination provides another example of this type of short-lived immunity. Although seroconversion to DENV1 was low after primary vaccination (12.1% seroconversion), after secondary vaccination about 70% of subjects had seroconverted. However, within 4 months after the second vaccination, ~40% of subjects remained seropositive and the residual geometric mean titer of DENV1-specific antibodies appeared to have declined to below a $PRNT_{50} = 10$ (52). Rapidly waning immunity after vaccination with a live, attenuated DENV vaccine is not unique to CYD-TDV since vaccination with a tetravalent PDK-attenuated DENV vaccine also elicited detectable antibody titers after two vaccinations that decayed rapidly and in some instances declined to below the cut-off value of 1:10 by the PRNT assay (45). Together, this illustrates the point that booster vaccination with live-attenuated CYD-TDV (and other DENV vaccines) is not only required to increase the breadth of serotype-specific neutralizing antibody responses but, similar to vaccines against other pathogens (e.g., MMR, acellular pertussis, varicella), booster vaccinations are important for inducing immune responses that can be maintained above a protective threshold for a prolonged period of time (53).

In contrast to these examples of non-protective or short-lived immunity, long-lived vaccine-mediated immunity can be achieved by at least two mechanisms; induction of an immune response that is long-lived and maintained at a plateau above the protective threshold (**Figure 1C**) or induction of immunity that may decline at a rapid rate but still be maintained above the protective threshold for a prolonged period of time if it begins at a high initial starting point (**Figure 1D**). Natural infection with measles or mumps is known to induce long-lived immunity that is often maintained above the protective threshold for many years or possibly for life (49). Vaccination with the live, attenuated yellow fever vaccine is also thought to induce life-long immunity (53). However, closer examination of published studies examining the durability of immunity following yellow fever vaccination indicates that it may reflect a combination of **Figures 1B** and **1C** since about 60–70% of vaccinated subjects maintain durable virus-specific neutralizing antibodies above the protective threshold (i.e.,

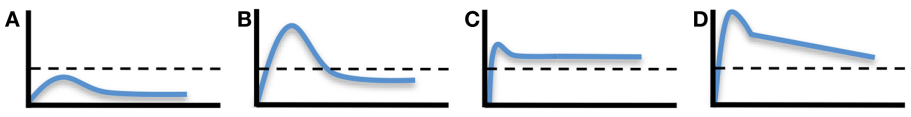

FIGURE 1 | Dynamics and duration of vaccine-induced immunity. The development of pathogen-specific immunity after vaccination can follow a number of different kinetic models. **(A)** Development of a measurable immune response can be determined, but if it does not reach above the protective threshold (indicated by the dashed line), then measurable immunity will note equate to protective immunity. Short-lived protective immunity **(B)** is common following primary immunization and is one of the reasons why most vaccines require booster vaccination. Long-lived protective immunity may be achieved by durable or nearly steady-state levels of immunity **(C)** or through the development of strong but rapidly declining immunity **(D)**, if the starting point begins high in reference to the protective threshold. It is important to note that the protective threshold will differ by pathogen or disease and an immunological correlate of protection must be known in order to extrapolate the potential durability of a particular vaccine-mediated immune response.

Figure 1C) whereas ~30–40% of vaccinees have neutralizing antibody titers that decline to below the protective threshold within 5–10 years after vaccination (i.e., **Figure 1B**) (53–55). Compared to most types of viral infection, the duration of immunity against tetanus is relatively short-lived with ~11-year half-life (49, 56). However, since the current five-dose tetanus vaccination regimen induces relatively high titers of tetanus-specific neutralizing antibody, protection is likely maintained above the protective threshold of 0.01 IU/mL for decades, despite having a more rapid decay rate over time (Slifka, manuscript submitted). There are several DENV vaccines under development (live/attenuated, inactivated whole virus, subunit envelope protein, and plasmid DNA) and it remains to be seen if these different approaches to DENV vaccination elicit different levels and duration of immunity. However, to get to the heart of the question pertaining to the duration of vaccine-mediated protection, the immunological correlate of protection to each DENV serotype will need to be identified.

DEFINING A CORRELATE OF PROTECTIVE IMMUNITY

A major caveat to the development of a vaccine against DENV is that the immunological correlate of protection against DENV infection is not currently known. It is possible that a titer of 1:10 may be readily detectable, but may still reside below the protective threshold needed to prevent infection or reduce DENV-associated disease in the clinical setting. Defining a correlate of protective immunity is important especially when examining the durability of vaccine-induced protection (**Figure 1**). If an appropriate animal model exists, then the correlate of immunity can be determined experimentally. For example, RM are highly susceptible to yellow fever and the correlate of immunity to this virus was first identified by vaccinating RM with graded doses of YFV-17D, followed 20 weeks later by infection with a lethal dose of virulent YFV-Asibi. Approximately 94% of vaccinated animals with a pre-existing log neutralizing index (LNI) of $\geq$0.7 were protected from lethal infection whereas 91% of animals with <0.7 LNI succumbed to yellow fever infection (57). In this case, RM develop disease that is similar to severe human yellow fever and this is likely one of the reasons why the correlate of immunity to yellow fever (LNI $\geq$ 0.7) has gained wide acceptance and has been used successfully in the field. Neutralizing antibodies are also believed to be the major component of vaccine-mediated immunity against DENV and protection against DENV viremia in non-human primates (NHP) is associated with a $PRNT_{50}$ titer of $\geq$10 (15). However, most of the DENV-vaccinated animals in this study (23/24; 96%) had neutralizing antibody titers that were $\geq$20 at the time of DENV challenge (note; 83% had $PRNT_{50}$ $\geq$40) and so the cut-off value of $PRNT_{50}$ $\geq$10 for defining protective immunity may still be an open question. Bearing in mind that DENV strains do not replicate to high titers or cause clinical disease in NHP, it is also difficult to extrapolate to the levels of immunity that might be required to protect against more severe human disease. As shown in the CYD-TDV Phase IIb vaccine trial, detection of a measurable level of antiviral antibodies (i.e., $PRNT_{50} = 10$) does not necessarily equate to a protective level of neutralizing antibodies (13).

To better understand the mechanisms of protective immunity against DENV in the absence of a robust animal model, a human challenge model of DF is being developed (32, 58–61). Well-characterized strains of DENV1 and DENV3 that were originally tested as vaccine candidates were found to elicit fever and mild dengue disease in early clinical trials and have now provided important information on DENV host–pathogen interactions (32, 58, 60) with the opportunity to directly test vaccine efficacy and determine immunological correlates of immunity. Following infection with DENV1-Ch (DENV1 45AZ5 PDK-0), both of the unvaccinated control subjects developed fever and dengue-associated illness lasting 2–6 days. In contrast, none of the five subjects who had previously received a live, attenuated tetravalent DENV vaccine showed disease symptoms. At the time of challenge, their DENV1-specific neutralizing $PRNT_{50}$ titers were 415, 235, 451, 198, and <10. This indicates that DENV vaccination can protect against DENV1-Ch, but in this small group of subjects there were no break-through cases lacking protection and it was therefore not possible to estimate a correlate of vaccine-mediated immunity against DENV1 disease. In another series of experiments involving experimental infection with DENV3-Ch (DENV3 CH53489 cl 24/28 PDK-0), both unvaccinated control subjects developed fever and dengue-associated illness and 3/5 TDV-vaccinated subjects also developed DF. The three vaccinated subjects who developed mild DF had pre-existing $PRNT_{50}$ titers of <10, 19, and 16, whereas the two vaccinated subjects who were protected from disease symptoms had pre-existing titers of 57 and 116. Based on these early results, this work would suggest that a DENV3-specific neutralizing titer of >20 or >50 may be necessary to protect against clinical DF. Further studies are needed to develop a model for DENV2 and DENV4 (60) and more vaccinated subjects may need to be enrolled with a range of pre-existing antibody titers in order to verify and refine the cut-off value for protection from DENV disease in this challenge model. Alternatively, another useful approach would be to coordinate large seroepidemiology studies of vaccinated subjects in DENV-endemic areas. If serum antibody titers were measured annually and cross-referenced to cases of clinical DENV illness/hospitalization, then it may be possible to extrapolate a vaccine-induced correlate of immunity that distinguishes between protective and non-protective immune responses. Once an immunological correlate can be determined for each of the DENV serotypes, then analysis of the neutralizing titers of DENV vaccines could be measured against this benchmark in order to better predict potential protective efficacy in Phase III trials.

CONCLUSION

Although an effective DENV vaccine has not yet reached the market, there are several candidates currently in clinical trial and it is likely that at least one or more of these vaccine platforms will provide protective immunity against this important, yet previously neglected disease. Although the current frontrunner, CYD-TDV, has yet to demonstrate effective protection in Phase IIb field trials, there is still hope that the Phase III trials will be successful. Because the CYD-TDV vaccine elicited only weak/partial immunity, there was concern that ADE would be a factor and one successful outcome of this study is that there was no evidence for exacerbated DENV disease among vaccinated children, which in itself is an important step forward in terms of identifying risk factors during DENV vaccine development. Despite induction of antiviral

T cell responses to both structural and non-structural proteins, flavivirus-specific T cell memory in humans appears to play a relatively subordinate role in protection against reinfection (7, 38, 62). Antiviral T cells may play a more important role during primary viral infection than during vaccine-mediated immune responses to secondary infection (7, 62) and it is likely that a specific level of serotype-specific neutralizing antibodies will be found as an immunological correlate of immunity following vaccination. Once an immunological correlate of vaccine-mediated protection is identified, then this will provide the opportunity for more quantitative assessment of current and future "next-generation" DENV vaccines in addition to providing a benchmark for determining the duration of protective immunological memory after vaccination.

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Epidemiological risk factors associated with high global frequency of inapparent dengue virus infections

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Dengue is a major international public health concern, and the number of outbreaks has escalated greatly. Human migration and international trade and travel are constantly introducing new vectors and pathogens into novel geographic areas. Of particular interest is the extent to which dengue virus (DENV) infections are subclinical or inapparent. Not only may such infections contribute to the global spread of DENV by human migration, but also seroprevalence rates in naïve populations may be initially high despite minimal numbers of detectable clinical cases. As the probability of severe disease is increased in secondary infections, populations may thus be primed, with serious public health consequences following introduction of a new serotype. In addition, pre-existing immunity from inapparent infections may affect vaccine uptake, and the ratio of clinically apparent to inapparent infection could affect the interpretation of vaccine trials. We performed a literature search for inapparent DENV infections and provide an analytical review of their frequency and associated risk factors. Inapparent rates were highly variable, but "inapparent" was the major outcome of infection in all prospective studies. Differences in the epidemiological context and type of surveillance account for much of the variability in inapparent infection rates. However, one particular epidemiological pattern was shared by four longitudinal cohort studies: the rate of inapparent DENV infections was positively correlated with the incidence of disease the previous year, strongly supporting an important role for short-term heterotypic immunity in determining the outcome of infection. Primary and secondary infections were equally likely to be inapparent. Knowledge of the extent to which viruses from inapparent infections are transmissible to mosquitoes is urgently needed. Inapparent infections need to be considered for their impact on disease severity, transmission dynamics, and vaccine efficacy and uptake.

Keywords: dengue, subclinical, inapparent, asymptomatic, infection

INTRODUCTION

Dengue has become a major international public health concern and is the most important arthropod-borne disease of humans (1–4). Any of the four antigenically distinct viruses, or serotypes, designated dengue virus (DENV)-1, DENV-2, DENV-3, and DENV-4, belonging to the *Flavivirus* genus in the family *Flaviviridae*, can cause dengue fever (DF), an acute viral infection characterized by fever, rash, headache, muscle and joint pain, and nausea, as well as more severe forms of the disease. A possible fifth serotype has recently been detected, but its global significance remains to be seen (5). Occasionally, DF progresses to dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), a potentially life-threatening illness associated with vascular leakage, hemorrhage, and shock. By contrast, it is increasingly recognized that the majority of DENV infections are subclinical, resulting in insufficient discomfort for clinical consultation (6). This reservoir of infection needs to be addressed.

Over the past decade, the number of dengue outbreaks has escalated (4), and the population at risk is increasing yearly. More than 3.5 billion people are at risk of DENV infection. It has recently been estimated that there are 390 million DENV infections every year, of which up to 96 million are symptomatic (7). This prolific increase has been associated with societal changes such as population growth and increasing urbanization, particularly in tropical cities with poor waste and water management, leading to proliferation of the domestic and peridomestic mosquito species that transmit DENV, *Aedes aegypti* and *A. albopictus*. Human migration and international trade and travel are constantly introducing new vectors and pathogens into novel geographic areas (8, 9). In addition, it has been suggested that rising temperatures and global climate change may lead to the expansion of the range of major mosquito vectors into new areas, extension of the transmission season in areas with currently circulating DENV, decrease in extrinsic incubation period, and increase in the mosquito spp.

vectorial capacity (10, 11). The potential threat of DENV invasion of continental Europe has recently been illustrated by cases of autochthonous dengue in southern France (12). These cases bear testament to the capacity of local *A. albopictus* mosquito vector to transmit the virus. This Asian tiger mosquito is the major potential vector of DENV in Europe, although the most important vector world-wide, *A. aegypti*, was identified in Madeira Island, Portugal, in October 2005. A major epidemic occurred in Madeira in 2012 (13). Autochthonous transmission of DENV in the United States has also been reported intermittently over the past decade in Texas, Hawaii, and Florida (14, 15).

International travel will ensure importation of virus into non-endemic countries from regions endemic for dengue. Infected individuals may harbor sufficiently high viral loads to infect mosquitoes prior to the onset of symptoms and thereby introduce the virus into the population. Potentially more important is the epidemiological significance of inapparent, subclinical infections. Travelers may import virus without showing overt clinical symptoms and thus will not be detectable either in the airports or once in the country. There is some suggestion that primary (1°) DENV infections can be majoritarily inapparent in certain outbreaks (16), whereas secondary (2°) infections lead to more severe symptoms even when occurring 20 years later (17). In fact, the longer the interval between heterotypic DENV infections, the higher the case fatality rate (18). The public health consequences of such inapparent infections are considerable, because apparently naïve populations may well have been previously exposed to infections, and once hospital cases of dengue are detected, the population as a whole may have already been primed with prior DENV infection.

The frequency of inapparent infections is extremely variable year to year, the risk factors poorly understood, and the terminology not standardized. Subclinical, inapparent, and asymptomatic infections are often used as synonyms, and the use of paucisymptomatic is used to designate a DENV infection with few symptoms. We will use subclinical and inapparent to denote infections with insufficient symptoms to be detected by the research or national surveillance program and/or to incite the infected individual to consult, but for which there is evidence, either by seroconversion or detection of virus, that the individual was infected with DENV. Asymptomatic infections will be used when there are no symptoms at all reported by the infected individual during an active infection, whether inferred by seroconversion or serology.

We review the literature on the extent of inapparent DENV infections, identify associated risk factors, and highlight several important lacunae that need to be addressed to assess the extent of the epidemiological importance of inapparent infections. We combine a PubMed literature search approach with review of articles cited within PubMed hits, plus a review of the classical pre-PubMed dengue literature. The search strategy was dengue + one of the following: inapparent, asymptomtic, subclinical. This search (27 January 2014) yielded 28, 151, and 34 articles, respectively. Acceptance criteria for selection were: (i) definition of symptom classification, (ii) ascertainment of symptoms and recent/concurrent viral infection at an individual level, and (iii) quantification of the number of inapparent infections. Of the retrieved PubMed articles, 33 publications fulfilled the acceptance criteria (19–51). An additional 14 articles cited within the above fulfilled acceptance criteria (52–65). One further recent article was known to the authors (66). A short description of each study is presented in the Supplementary Material and in **Table 1**. Below, we highlight features pertinent to dengue epidemiology that these studies elucidate.

RETROSPECTIVE AND OUTBREAK STUDIES

There were 12 retrospective or outbreak studies with measures of seropositivity and subjective recollection of fever and/or dengue-associated symptoms (Australia 1, British Virgin Islands 1, Colombia 1, Cuba 2, Puerto Rico 2, Brazil 2, South Pacific 1, Singapore 1, and Taiwan 1) (19–25, 53–57). In Cuba, the inapparent rate during the 1981 DENV-2 epidemic was estimated to be 71% in whites and 88% in blacks; the infections were considered likely to be 2°, as 45% of the population was thought to have seroconverted during the 1977 DENV-1 epidemic (54). During the 1997 Cuban DENV-2 epidemic, all 2° infections were clinically overt, but only 3–6% of 1° infections were apparent (55). In the Brazilian studies, inapparent rates were 27 and 53% in 1° infections vs. 37 and 39% in 2° infections (22, 23). In Colombia, repeated cross-sectional studies were carried out over a period of 17 months; 259 of 3,189 individuals showed clinical signs of viral infection and/or anti-DENV IgM antibodies; 86% were inapparent (24). In the Puerto Rican studies, where infections were majoritarily 1°, inapparent rates were 53 and 43% (56, 57). The Singapore and South Pacific studies were carried out during the epidemic period, and inapparent rates of 78 and 60% were reported, respectively (25, 53). The Australian study was performed in 1995 to address the 1993 DENV epidemic; only 11% of infections were considered to be inapparent (19). Longer-term recollection of 1° infections in individuals hospitalized in Taiwan with 2° infections suggested that 80% of 1° infections had been inapparent (21). Finally, returning US volunteers from the British Virgin Islands in a community with a suspected dengue case revealed that all DENV IgM-positive individuals had recollection of symptoms (20).

NON-RESIDENTS (EXPATS, MILITARY, AND TRAVELERS)

Nine prospective studies were identified involving expats, travelers, or military personnel staying in dengue-endemic areas (Haiti 1, Singapore 1, Somalia 1, Thailand 2, various 4) (48–51, 61–65). Seroconversion rates were low, yielding relatively few infections in any study. In the majority of studies, symptomatic infections referred to the occurrence of any dengue-like symptom. Sharp et al. (62) and Cobelens et al. (49) defined a symptomatic infection as fever plus any other symptom. Baaten et al. (50) obtained objective measure of fever or any other symptoms for defining symptomatic DENV infections (50). For the majority of individuals, the infection was considered to be their first, and the inapparent rate ranged from 0 to 100%. The majority (80–100%) of Americans (61) and Japanese (48) in Thailand had symptoms, whereas no Australian travelers to Asia reported symptoms (51). Sixty to 80% of Dutch travelers (world-wide) reported no symptoms (49, 50), whereas 50% of Israeli travelers had symptoms (63). Over 90% of Chinese workers experienced symptoms in Singapore (64), 85% of American military personnel had symptoms in Somalia (62), and all seven missionaries who were seropositive for DENV returning from Haiti reported dengue-like symptoms (65).

Table 1 | Prospective studies addressing the inapparent rate.

Place and reference	Virus (years)	Minimum symptom	% Inapparent (*N*)	Incidence rate: disease; infection	Age (years)	Seroprevalence
Puerto Rico (58)	DENV-4 (1982)	Fever	45 (56)	36%; 31%	All	70%
Nicaragua (59)	DENV-1 and DENV-2 (2001–2003)	Fever	91 (106)	8.3–8.5/1000; 6–12%	4–16	91%
Nicaragua (39, 41, 42)	DENV-1 (2004–2005); DENV-2 (2006–2008); DENV-3 (2008–2010)	Fever or WHO	83 (1778)	3.4–43.5; 67–110 (/1000 person-years)	2–14	30% age 2; 90% age 9
Nicaragua (40)	DENV-2 (2006–2007)	Any	42 (12)	0.2–1.4%; 7%	11–67	ND
Peru (52)	DENV-1 and -2 (1999–2001); DENV-3 (2002–2028); DENV-4 (2008–2011)	Fever	93 (3837)	0.5–19; 2–90 (/100 person-years)	All	60% age 2; 90% age 15
Brazil (38)	DENV-2 and DENV-3 (2007–2008)	Fever+ 2 WHO	77 (30)	2%; ND	1–79	56–77%
Thailand (26)	DENV-1–4 (1980–1981)	Fever	87 (103)	0.74%; 5.9%	4–16	39% 4–6 years; 73% 13–15 years
Thailand (27)	DENV-1–4 (1998–1999)	Fever	54 (331)	2.7%; 5.8%	7–11	ND
Thailand (30)	DENV-1–4 (1998–2002)	Fever	56 (569)	6.4%; 25.5%	7–15	ND
Thailand (45)	DENV serotype not known (2000–2001)	Fever	85 (34)	2; 7 (/100 person-years)	Birth – 8	ND
Thailand (32)	DENV-4 (2004–2005) and DENV-1 (2006–2007)	Fever	65 (535)	1.3–4.4%; 6.9%	4–15	ND
		Fever or other	20 (119)	11.8%; 16%[a]	0.5–15	
Thailand (29)	DENV-4 (2004–2005)	Fever	52 (27)	6.0%; 12.4%[a]	0.5–15	ND
Thailand (60)	DENV-1 (2001)	Clinical consultation	94 (54)	0.2%; 3.1%	All	6%
Thailand (28)	DENV-1 (2000–2001); DENV-2 (2002)	Clinical consultation	91 (733)	0.3%; 1–27%	All	ND
Vietnam (35)	DENV-2 (2004–2005); DENV-1 (2006–2007)	Fever ≥38° for ≥2 days	80 (953)	17–40/1000; 8–14%	2–15	20–29%
Vietnam (44)	DENV-1 dominant	Fever	90 (10)	0.2; 1.7 (/100 person-years)	Birth – 2	ND
Indonesia (37)	DENV-1–4 (2001–2003)	Fever	47 (17)	1.5%; 2.5%	All	ND
Indonesia (36)	DENV-1–4 (2000–2002)	Any	75 (74)	18; 74 (/1000 person-years)	18–66	ND
Philippines (46, 47)	DENV-3 and DENV-2 (2007–2009)	Clinical consultation	90 (115)	8–16; 103 (/1000 person-years)	Birth – 1	ND
Multi-centric (43)	DENV-1, -2, -3 (2006–2007) Southeast Asia	Fever	85 (20) Southeast Asia	0.9%; 6.1%[b]	>24 months	ND
	DENV-1, -3 (2006–2007) Latin America		63.2 (19) Latin America	8.3%; 22%[b]		

[a] *Indicates positive index clusters only; cluster contacts followed for 15 days or*

[b] *7 days.*

BIRTH COHORTS – MATERNAL ANTIBODY STUDIES

Four birth cohort studies addressed the potentially deleterious effects of maternal antibody for outcome of DENV infection in infants (Philippines 2, Vietnam 1, Thailand 1) (44–47). The occurrence of severe disease in infants following their first infection was noted by Halstead and colleagues and contributed to the development of the theory of antibody-dependent enhancement (ADE); antibodies from a first infection are insufficient to neutralize virus from a second infection of a different serotype and actually increase virus internalization in Fcγ receptor-bearing target cells and hence viremia (67). In infants, following a period of protection by maternally acquired anti-DENV antibodies, catabolism of these antibodies was hypothesized to decrease the titer of maternally acquired antibodies to enhancing levels and thus lead to the high incidence of severe dengue disease observed in infants (68). The birth cohort studies did not confirm the ADE hypothesis, although samples sizes were small. The inapparent DENV infection rate ranged from 75 to 90%.

PROSPECTIVE STUDIES

Twenty-three published papers describe analyses of prospective studies carried out in Southeast Asia (Thailand 10, Vietnam 1, Indonesia 2) (26–37, 60) and the Americas (Brazil 1, Nicaragua 5, Peru 2, Puerto Rico 1) (38–42, 52, 58, 59, 66) and one multicenter study covering Vietnam, Cambodia, French Guiana, and Brazil (43). The Thai studies occurred in one of three sites (Bangkok, Chang Mai, or Kamphaeng Phet); the Nicaragua, Peru, and Indonesia studies occurred in Managua, Iquitos, and Jakarta, respectively. The studies used one or more of several protocols: community-based cohort with paired healthy samples and laboratory work-up of suspected dengue cases/undifferentiated febrile illnesses, follow-up of school-based absenteeism, and index cluster analysis. Anti-DENV antibodies were detected using rapid diagnostic kits, in-house ELISA assays, hemagglutination inhibition assay, and/or neutralizing antibody tests. The age group sampled varied considerably, as did the seroprevalence and force of infection. Notably, although the majority of studies focused on children or the general community, one of the Indonesian studies targeted an adult cohort (36). The classification of a symptomatic DENV infection varied from the presence of any symptom, to just fever or fever plus two additional dengue-associated symptoms according to the WHO case definition. The inapparent DENV infection rate ranged from 20 to 97%; the weighted mean inapparent rates of cluster and cohort studies were 37 and 76% respectively. Mean inapparent rates in cohort studies were 77.1% in the Americas and 74.4% in Asia.

EXPERIMENTAL INOCULATIONS (Pre-1960s)

The experimental infection studies in the first half of the twentieth century provide the foundations of our current knowledge of dengue (69, 70). Siler et al. (69) conducted a series of induced infection experiments in military personnel using infected mosquitoes. Of 47 subjects, four individuals remained refractory to infection (or were asymptomatic) and two had very mild symptoms. The inclusion criteria aimed to recruit individuals who were naïve to dengue, but it could not be ruled out that some individuals had been previously exposed to DENV. Thus, assuming no immunity, the inapparent rate was at most 13% (6/47).

Simmons et al. (70) gave detailed accounts of the course and outcome of infection using mosquito-induced infections in American military personnel, residents of the Philippines, monkeys, and other animals (70). All 81 infections induced using competent species of mosquitoes (*A. aegypti*, *A. albopictus*) after an appropriate extrinsic incubation period (>9 days) yielded DF; only 13.6% were classified as mild (undefined). The extent of asymptomatic or even inapparent infections was clearly very low in American military personnel with no likely previous exposure to dengue. Adult Philippine individuals living in endemic zones for dengue proved immune, whereas those from non-endemic areas proved susceptible and were symptomatic following DENV infection. Successful experimental infections without symptoms in naïve Macaque monkeys were achieved, as demonstrated through onward transmission to mosquitoes. Although such onward transmission studies were not carried out in purportedly unsuccessful DENV-induced infections in humans, the authors state "In addition, it is quite probable that mild unrecognizable infections may occur in many adults, as has been proved possible in monkeys, and that virus can be transmitted from these apparently symptomless cases of dengue."

POTENTIAL EXPLANATIONS FOR DIFFERENCES IN OBSERVED INAPPARENT RATES

DETECTION METHODOLOGY

Retrospective surveys involving questionnaires of perceived symptoms are open to perception bias as well as the non-specificity of dengue symptoms. Prospective studies use varying definitions of a symptomatic dengue episode and different protocols for case detection that generate considerable variation in inapparent infection rates. This is particularly well demonstrated by two alternative protocols implemented in the same population: the index cluster approach revealed that many inapparent infections, as defined by school absenteeism and passive case detection, had fever or other symptoms (32). In addition to the increased case detection sensitivity of the index cluster approach, such an approach may also suffer from ascertainment bias: viruses responsible for index cases identified by clinical presentation may be more pathogenic and thus lead to increased symptomatic infections in the clusters than would occur in the general population. However, symptomatic cases in clusters were found to be milder than those in the cohort study (33).

INFLUENCE OF HUMAN GENETICS

A broad overview of global incidence of disease attributable to dengue suggests that disease severity is greater in Southeast Asia than in the Americas and that severe dengue is infrequent in Africa (71). One major confounding factor is separating geography and the environment from ethnicity. However, the dengue epidemics in Cuba have given support to the hypothesis that individuals of African ethnicity are less susceptible to disease than white Caucasians (54). There is increasing evidence from candidate gene and genome-wide studies that human genetics play a role in the outcome of infection (72–74). Only one study, however, has attempted to assess the impact of human genetics on inapparent outcome

of infection. A polymorphism in the FcγRIIA was found to be associated with inapparent infection vs. DF or DHF in the Cuban population (75). In light of the epidemiological observations on the global variation in the incidence of DENV infection and severe disease, it seems likely that at least some of the observed variation in the inapparent rate is attributable to human genetics.

PRIMARY VS. SECONDARY VS. POST-SECONDARY INFECTIONS

Secondary infections are considered to result in more severe outcome of infection, due to the phenomenon of ADE and/or cross-reactive T cells (67). Very little, however, is known specifically about the impact of previous exposure to two serotypes on the outcome of infection with a third serotype. A cohort study in Brazil found that there were significantly more inapparent infections in 1° as compared to 2° infections (22). Olkowski et al. (66) found that in Peru there was a reduction in symptomatic outcome in post-secondary infections compared with 1° and 2° infections as defined by pre-infection serological profile; 93% reduction in symptomatic outcome for DENV-3 and a 64% reduction in disease outcome for DENV-4 (66). However, Montoya et al. (42) found no differences in inapparent infection rates in first, second, third, or even post-secondary infections in Nicaragua (42). Several studies measuring the inapparent rate also evaluated whether the observed infection was 1° or 2°(22, 23, 26, 29, 32, 38, 39, 42, 43, 52, 59). A mixed model logistic univariate regression revealed that although there were significant differences in inapparent rate among studies, there was no significant difference within study site between the inapparent rate in 1° and 2° infections (Wald's $\chi_1^2 = 0.27$, $P = 0.61$) (**Figure 1**). Too few studies have been able to address post-secondary (third or fourth) infections and infection outcome for any meta-analysis to be performed.

SHORT-TERM CROSS-PROTECTIVE IMMUNITY

Sabin set the foundations for our current appreciation of acquired immunity to dengue and the extent of cross-immunity (76). Three important results arising from these early studies are of pertinence here: (i) there exists a minimum infective dose, which could lead to no symptoms but partial immunity; (ii) immunity to a recent previous infection alters the outcome of a subsequent infection; and (iii) virus attenuated via mouse passage yields symptomless infections that are transmissible to mosquitoes, albeit poorly so. For cross-immunity, active immune protection was achieved for up to 2 months, slight malaise/fever occurred in 2° infections 2–3 months later, and even after 9 months, dengue episodes were milder. DENV infection and onward transmission to mosquitoes was demonstrated in 2° infections at 2–3 months and 9 months post-1° infection. Recent statistical and theoretical modeling approaches lend support to Sabin's demonstration that there exists cross-serotype non-sterilizing immunity resulting in milder clinical symptoms that may last for up to 2 years (77–79).

Endy et al. (30) first noted a significant impact of the previous year's dengue incidence on the inapparent rate; a high incidence the previous year increased the current year's inapparent rate. This was proposed to be a result of heterotypic cross-immunity, as described above. A plot of the inapparent rate against previous dengue incidence reported in the longitudinal cohorts with sufficient data (Nicaragua, Peru, Thailand, and Vietnam) (30, 35, 39, 41, 42, 52), all show the same positive relationship between the incidence of infection the previous year and the inapparent rate in the current year (**Figure 2**). However, the strength of the relationship seems proportional to the seroprevalence. In Nicaragua and Peru, the seroprevalence in the population was high, with most children having been exposed to at least one serotype by 10 years

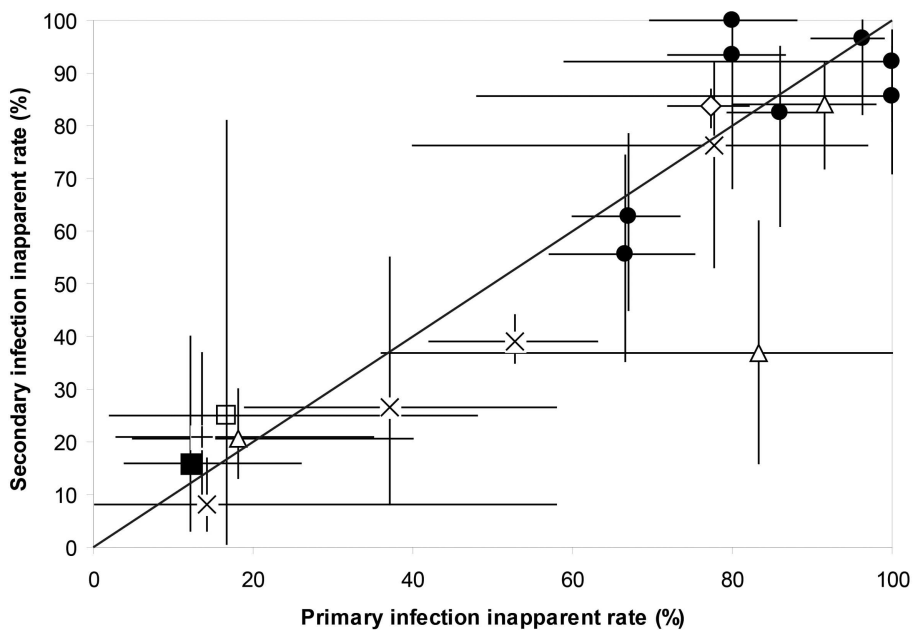

FIGURE 1 | The inapparent rate of primary and secondary DENV infections within the same study site for Nicaragua (39, 41, 42, 59**), Thailand (**26, 29, 32**), Brazil (**22, 23, 38**), Peru (**52**), French Guiana, Cambodia, and Vietnam (**43**)**. Shown are means and 95% binomial confidence intervals. Superimposed is the line of equality where the inapparent rate of 2° infections equals that of 1° infections. • Nicaragua, ■ Vietnam, + Cambodia, × Brazil, ◇ Peru, □ French Guiana, △ Thailand.

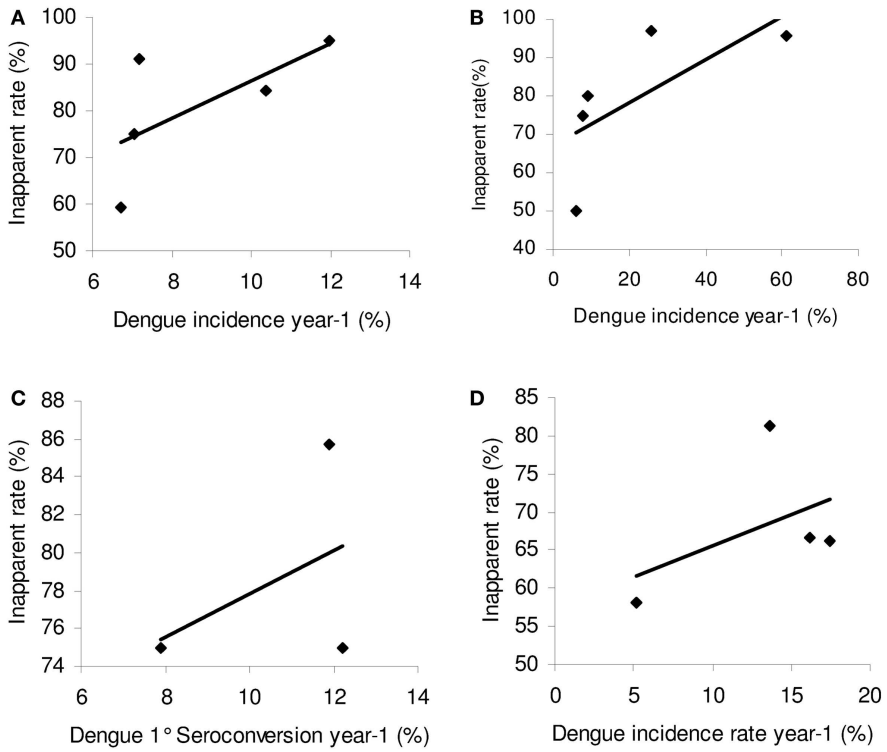

FIGURE 2 | The relationship between the inapparent infection rate and DENV infection intensity the previous year. Linear trend lines were fitted. **(A)** Nicaragua (39, 41) $R^2 = 0.43$; **(B)** Peru (52) $R^2 = 0.477$. **(C)** Vietnam (35) $R^2 = 0.20$; **(D)** Thailand (30) $R^2 = 0.21$.

of age. In Vietnam, by contrast, the seroprevalence was lower for the same age group (35). The correlation of the incidence of infection the previous year and the inapparent rate in the current year are strongest for areas of higher seroprevalence and thus where the majority of new infections are 2°. It is notable that the positive relationship for inapparent rate and previous year DENV incidence of infection was significantly stronger when considering incidence of disease the previous year ($R^2 = 0.76$) rather than infection ($R^2 = 0.2$) in the Vietnam cohort. This raises the question of whether the acquired immune response is stronger when infection is accompanied by disease, rather than being asymptomatic; this has previously been observed in Japanese Encephalitis (quoted in Barnes and Rosen) (53).

Of key importance is to ascertain whether the inapparent rate is indeed driven by heterotypic immunity. The Nicaraguan Pediatric Dengue Cohort Study throws some light on the question, differentiating 1° from 2° infections (39). A reduced symptomatology in the outcome of infection through heterotypic immunity would only apply to 2° infections. The fluctuations in the inter-annual inapparent rate in 2° infections oscillated inversely with the previous years' incidence rate, ranging from 67 to 97% and significantly so with non-overlapping 95% binomial confidence intervals (CIs) (calculated as inapparent/total infections) (**Figure 3B**). By contrast, although the same oscillating inter-annual pattern was observable in 1° infections, for which cross-protective immunity is not relevant, the fluctuations were dampened, ranging from 82 to 92%, with overlapping 95% CIs (**Figure 3A**). This gives credence to the hypothesis that short-term cross-protective immunity plays a significant role in determining infection outcome in 2° infections.

More recently, several studies have analyzed the importance of the time interval between successive DENV infections on the outcome of infection in first, second, and post-2° infections. In the Nicaragua cohort, Montoya et al. (42) found that the time interval between successive first and second infections leading to an inapparent second infection outcome was significantly shorter (1.8 years) than that leading to a symptomatic infection outcome (2.6 years) (42). There was no impact of time interval for post-2° infections. In Thai cohorts, Anderson et al. (34) found similar results; there was a higher probability of an infection being inapparent in 2° infections if occurring within 1.4 years of a previous (thus 1°) infection; the time interval between infections leading to DF or DHF was longer, at 1.9 and 2.6 years respectively, although the number of DHF was small (34). Again there was no difference for post-2° infections.

IMPACT OF THE CURRENT YEAR'S INCIDENCE

Endy et al. (30) also noted a significant negative impact of the current year's dengue incidence on the inapparent rate; a high concurrent incidence reduced the inapparent infection rate (30). This relationship was confirmed in the same cohort ($R^2 = 1$) (32), but to a much lesser extent in both the Nicaraguan and Vietnamese studies ($R^2 = 0.12$ and 0.23, respectively) (35, 39). There was no relationship in the Peruvian study ($R^2 = 0.07$) (52). Careful studies

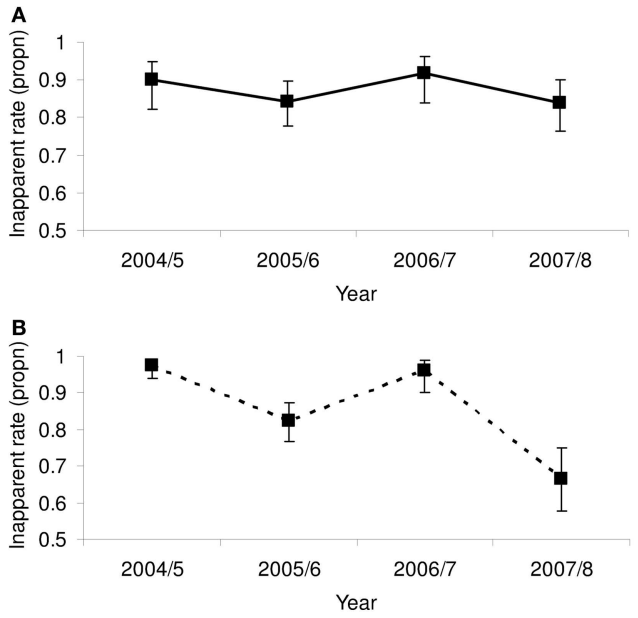

FIGURE 3 | The proportion of (A) 1° and (B) 2° infections in the Nicaraguan Pediatric Dengue Cohort Study (39) that was inapparent. Shown are the proportions and the 95% binomial confidence intervals.

taking into account the force of infection in relation to inapparent and symptomatic infection are needed.

The interplay between short-term cross-protective immunity and spread of a novel serotype will lead to inapparent rates that will depend on the historical prevalence of dengue (thus, the extent of 2° infections), the recent incidence of dengue (cross-protection), and the nature of the virus itself. By example, in the Nicaraguan cohort, the expansion of DENV-2 in 2005 (increasing from 20% of infections to 53%) was accompanied by an increase in transmission intensity (8.6–11.1% incidence) and increase in symptomatic outcome, particularly for 2° infections (3–25%) (39). This suggests a classic reaction to a new serotype in a background primed by other serotypes. The following year, transmission intensity dropped (from 11.1 to 5.8%), there was an increase in the predominance of DENV-2 (90% of all infections), and a decrease in rate of symptomatic infections; this decrease was most dramatic for 2° infections (25 to 4%), suggesting an important influence of cross-protective immunity. Then, transmission intensity increased with a concomitant rise in disease severity in both 1° and 2° infections. The rise in disease severity despite no change in serotype would suggest that the virus had evolved. Indeed, there was a clade change in 2006 (from DENV-2 clade 1 to clade 2B), which was associated with increased severity in the cohort and which could have contributed to the final increase in severity of infection outcome (80).

Morrison et al. (52) proposed a three-step chronology for the invasion of a novel serotype: amplification, replacement, and epidemic (52). Implicitly underlying this chronology is the notion of viral adaptation to its novel environment in competitive circumstances. Viral evolution may also contribute to the rise in the inapparent rate following the epidemic phase. Abortive dengue epidemics, where the incidence of disease is low, have been noted previously, and islands in the South Pacific have escaped severe outbreaks occurring in their neighbors (53, 81).

VIRAL GENETICS

The importance of viral genetics in determining the outcome of infection has been suggested in the context of 1° vs. 2° infections (82), severe primary epidemics in naïve populations (53, 81), molecular variants yielding high replication rates in the laboratory (83), DENV serotype, genotype, and clade, sequential order of serotypes in infections (84), and interaction with pre-existing serotype-specific immunity (80, 85). However, there is no consistent pattern. Whilst confounding factors influence the disease severity of an epidemic, the genetic diversity of the viruses likely plays an important role. As with all arboviruses, the RNA-dependent RNA polymerase's lack of proofreading activity coupled with the large virus population sizes lead to the constant generation of variants. This means that RNA viruses generally circulate as dynamic mutant networks (86). Recent studies reveal that DENV exist as heterogeneous populations in patients and mosquito vectors (87–93), but the significance of this is unclear. Coupled to the selective forces in both host and vector, these features would enable the circulating viral population to change significantly even during the course of a single epidemic. Indeed, the proportion of severe cases has been reported to increase toward the end of an outbreak (94–96). Endy et al. (27) also noted that symptomatic cases extended later into the season than inapparent infections (27). DENV populations may also rapidly change because of periodic selective sweeps and by intra-serotypic recombination (97, 98), though this latter remains contentious. DENV infection leads to a spectrum of outcome severity from inapparent to mild or severe disease; small changes in viral genetics could lead to significant changes in infection outcome. However, despite increasing evidence for a role of viral genetics in the outcome of infection, to date we do not know the extent to which the observed variation in the inapparent rate is influenced by viral diversity and evolution.

Genetic variation has been associated with differences in virus transmission efficiency (99, 100). Viral adaptation during its invasion phase may improve transmission capacity to mosquitoes responsible for an epidemic and/or result in strains that are responsible for more severe cases toward the end of the epidemic. Infectiousness to mosquito increases with viremia and although hospitalized cases have higher viremia, symptomatic but ambulatory cases infected mosquitoes equally well (101). Currently, we have no knowledge about the comparative transmissibility of inapparent infections, a crucial element that needs to be addressed.

CONCLUSION AND IMPLICATIONS

Establishing risk factors and the extent to which DENV infections are inapparent is important not only for assessing whether there will be a silent invasion of DENV into hitherto unaffected areas but also for improving our understanding of dengue epidemiology and infection severity. The epidemiological evidence to date suggests that whilst the majority of infections are inapparent in endemic settings, there are recognizable patterns that are consistent with an important role for short-term heterotypic non-sterilizing immunity. However, similar inter-annual fluctuations in the inapparent rate in 1° infections require additional explanations, potentially suggesting a role for viral evolution. One intriguing avenue of

research is the extent to which heterotypic immunity promotes viral diversification. Likewise, it would be interesting to assess how human genetics impacts upon infection outcome.

Extrapolating from endemic settings to an invasion scenario may not be applicable, especially given the significant role seemingly played by the immune response in 2° infections, whether enhancing or protective. Retrospective serological surveys in recent virgin soil epidemics, for example, in Madeira and Cape Verde, would provide invaluable information on the extent of inapparent infections under such scenarios and give a better idea of what to expect under invasion and hence how best to implement surveillance and control efforts.

Finally, an appreciation of inapparent DENV infections is important for both interpretation of vaccine trials and vaccine uptake. It has been suggested that, in the light of the high incidence of inapparent DENV infections, vaccine trials should consider other measures of vaccine efficacy in addition to occurrence of clinically apparent infections, i.e., to consider efficacy against infection and not just disease (102). Moreover, pre-existing immunity from prior symptomatic and inapparent DENV infections will likely affect the type of immunity induced by tetravalent vaccines (i.e., homotypic vs. heterotypic), and this needs to be considered as well. Lastly, successful vaccination should reduce the large reservoir of inapparent infections that are likely capable of onwardly transmitting the virus, thus further reducing DENV transmission.

AUTHOR CONTRIBUTIONS

All authors contributed equally to the manuscript. Specifically, Richard Paul and Eva Harris conceived the study. All authors participated in the data acquisition and preliminary writing. Etienne Simon-Loriere created the graphics and Richard Paul carried out the statistical analyses. Richard Paul and Eva Harris wrote the final version and all authors read, corrected, and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fimmu.2014.00280/abstract

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Human to mosquito transmission of dengue viruses

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The successful transmission of dengue virus from a human host to a mosquito vector requires a complex set of factors to align. It is becoming increasingly important to improve our understanding of the parameters that shape the human to mosquito component of the transmission cycle so that vaccines and therapeutic antivirals can be fully evaluated and epidemiological models refined. Here we describe these factors, and discuss the biological and environmental impacts and demographic changes that are influencing these dynamics. Specifically, we examine features of the human infection required for the mosquito to acquire the virus via natural blood feeding, as well as the biological and environmental factors that influence a mosquito's susceptibility to infection, up to the point that they are capable of transmitting the virus to a new host.

Keywords: dengue virus, transmission, *Aedes aegypti*, *Aedes albopictus*, viral titer, temperature, symptomatic vs. asymptomatic infections

INTRODUCTION

THE GLOBAL DENGUE BURDEN

The medical (1) and economic (2–7) burden of dengue is large; a recent probabilistic estimate suggested 100 million symptomatic cases occurred in 2010 (8). Human travel patterns are changing, and there is far more international traffic between dengue endemic countries and those that are usually dengue-free, albeit permissive for epidemics because of the presence of a suitable vector (9–11). This is evidenced by recent autochthonous dengue virus (DENV) transmission in Europe (12, 13) (local transmission subsequent to importation). The current scale of the public health problem of dengue highlights the need to better understand the underlying biological and environmental factors that result in human to mosquito transmission of DENV. A better comprehension of how these factors vary, and under what conditions, will help us to develop more effective interventions of DENV transmission.

HUMAN TO MOSQUITO TRANSMISSION OF DENGUE

Transmission of DENV from the human host to mosquitoes requires multiple biological factors to align in time and space. Under natural conditions, a susceptible mosquito can only acquire a DENV infection after it has taken a blood meal from a viremic person. When viremic blood arrives into the mosquito midgut, extracellular virus binds to undefined receptors on the cellular surface of the midgut epithelium. If the virus can successfully infect and replicate within midgut epithelium cells then new progeny virus are shed into the hemocoel (the cavity in which the hemolymph circulates, part of the open circulatory system of invertebrates), where it can subsequently disseminate and infect secondary tissues, including the salivary glands. Once sufficient virus replication has occurred in the salivary glands and upon the next probing/feeding event, the virus may be transmitted to a new host via the saliva of the infected mosquito.

VECTORS OF DENV

The primary vector of DENV is *Aedes aegypti*, an endophilic mosquito, preferring to live in and around homes in tropical and subtropical regions. This mosquito feeds preferentially on human blood under field conditions (14), and inhabits tropical and subtropical climates, with the geographic range spanning all continents except Antarctica. A secondary dengue vector, *Aedes albopictus*, is more exophilic under natural field conditions, commonly living outdoors, but still feeds almost exclusively on humans in Thailand (14), and preferentially on humans in the Indian Ocean (15). The strong preferences for human blood exhibited by these mosquitoes increase the potential for disease transmission among humans.

The expanding geographical range of DENV vectors (16, 17) underscores our need to examine DENV transmission dynamics in more detail. In the United States there has been a resurgence of *Aedes aegypti* across the South Eastern seaboard, and its presence has been noted as far north in California as South San Francisco Bay (W. K. Reisen and M. V. Armijos, UC Davis, personal communication, August 2013). *Aedes aegypti* is also expected to spread beyond its current range within Australia, which is presently throughout the state of Queensland, extending into the North Eastern part of Northern Territory (18). Autochthonous cases of dengue occurred in Portugal (Madeira Islands) in 2012 (13), with transmission attributed to the invasion of *Aedes aegypti* in the mid 2000s.

Aedes albopictus, an aggressive, nuisance day-time biter (19), is now established in numerous areas of Southern Europe (20–23), with its geographic range having continuously expanded since its first observation in Albania in 1979 (24). *Aedes albopictus* has also become established in parts of South America and Africa that were previously free of the invasive pest (16) (and references therein). Its emergence in Australia is also a significant threat (25). This (potential and actual) range expansion of *Aedes albopictus*,

particularly because it inhabits a more temperate environment than the tropical *Aedes aegypti*, may lead to an increased risk of DENV transmission as it brings a greater number of dengue-susceptible people into contact with vectors. Photoperiod-induced diapause and non-desiccating, cold-tolerant eggs further allows *Aedes albopictus* to survive in cooler environments for periods of the year (26, 27).

Other Aedine species have been shown to be capable of transmitting DENV under experimental conditions (28–30), including *Aedes polynesiensis*, *Aedes scutellaris*, and *Aedes japonicas*. As discussed in Rosen et al. (28), *Aedes polynesiensis* has been implicated in the natural transmission of DENV, but the relative contribution of each of these mosquitoes to overall transmission has not been quantified, and is thought to be negligible (31).

HUMAN FACTORS INFLUENCING TRANSMISSION

Factors that influence the transmission of DENV from humans to mosquitoes include the following.

VIRAL TITER IN HUMAN PLASMA

The amount of virus circulating in the blood of an infected human will influence the likelihood of a mosquito becoming infected after a blood meal. Nguyen et al. (32) identified the viremia characteristics in dengue cases that led to DENV infection of blood-fed *Aedes aegypti*. The viremia in humans required to infect 50% of mosquitoes differed between serotypes (**Figure 1**). The 50% mosquito infectious dose was ~10-fold lower for DENV-1 and DENV-2 (6.29–6.51 log10 RNA copies/ml) than for DENV-3 and DENV-4 (7.49–7.52 log10 RNA copies/ml). A dose–response relationship was observed such that with an increasing number of DENV RNA copies, there was an increased likelihood of a mosquito becoming infected, up to the point of saturation. These findings define the viremia level that interventions such as vaccines and antivirals must target for prevention or amelioration to reduce DENV transmission.

DURATION OF HUMAN INFECTIOUSNESS

Accumulated data from empirical infection studies on human subjects conducted in the first half of the twentieth century showed that humans can be infectious to mosquitoes from 1.5 days prior to the onset of symptoms to around 5 days after the commencement of symptoms (**Figure 2**) (33–37). In each of these studies, however, the assignment of the day or hour of the mosquito

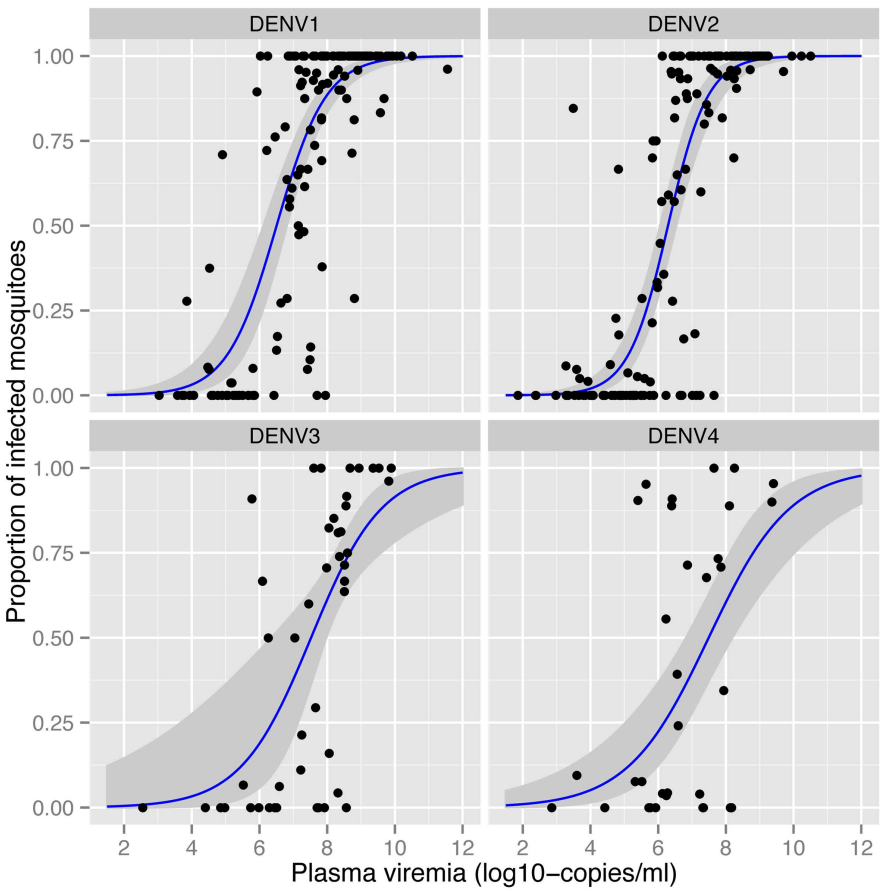

FIGURE 1 | Effect of plasma viremia on mosquito susceptibility to infection (32). With an increasing concentration of DENV in the patient's blood, mosquitoes have a higher probability of being infected, as determined 12 days after mosquitoes imbibed the blood meal. Each data point represents the proportion of DENV-infected mosquitoes after a single blood-feeding episode. Estimated associations and the 95% confidence intervals are shown in the blue lines and gray shading, respectively. Image reproduced with permission from the authors.

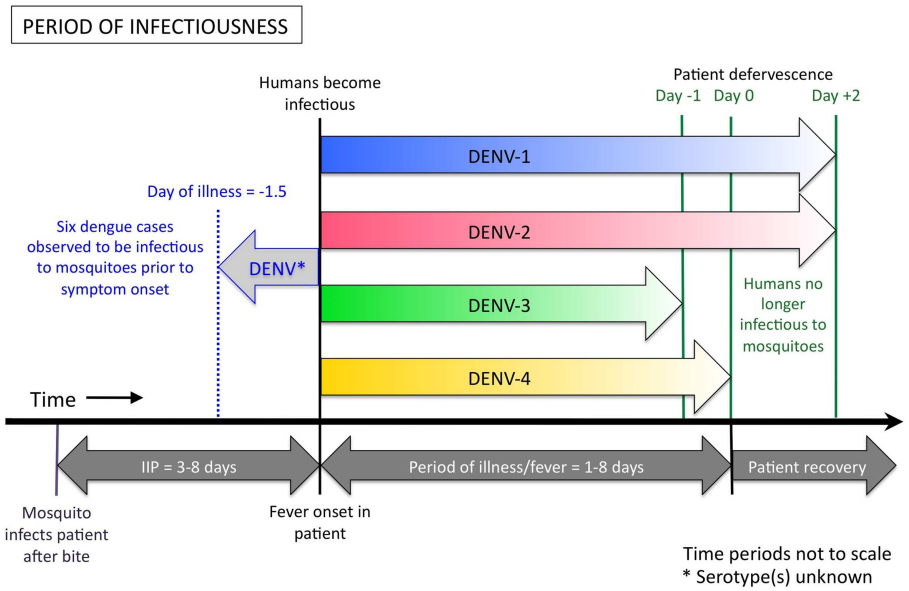

FIGURE 2 | Duration of human DENV infectiousness to *Aedes aegypti* mosquitoes. A person can become infectious to mosquitoes up to 1.5 days before the onset of their fever and associated symptoms, and remain infectious until the end of their febrile period, and sometimes shortly after. Indicated above the thick black line are the colored arrows showing the period of human infectiousness for each serotype, according to Nguyen et al. (32). DENV-1 (shown in blue) and DENV-2 (red) may be infectious to mosquitoes for up to 2 days after the patient becomes afebrile, however DENV-3 (green) and DENV-4 (yellow) appear to be less infectious at these later stages of illness, due to lower plasma viremia in the patient. A single study (37), found that six dengue patients were infectious to mosquitoes from 0.25 days, up to a maximum of 1.5 days, before they had any sign of symptoms (indicated by the pale gray arrow). The serotype of virus used in Siler et al.'s study is unknown. Below the black line is the relative alignment of the course of dengue illness. IIP = intrinsic incubation period.

exposures was not systematic [e.g., Cleland et al. (34) exposed mosquitoes to patients on the 18th, 22nd, 46th, 47th, 57th, 67th, and 90th hours after the onset of fever (with no apparent pattern or rationale behind the selection of these time points)], resulting in a broad range of exposure time points but with large gaps in between. In Nguyen et al.'s (32) more recent study, 208 patients who presented to the Hospital for Tropical Diseases in Ho Chi Minh City, Vietnam were enrolled in the study and randomly assigned to 2 days on which they would be exposed to naïve mosquitoes. Days of exposure ranged between day 1 and day 7 of illness. Results demonstrate that a small number of mosquitoes can still become infected with each of the four DENV serotypes up to the sixth day after illness onset. No mosquitoes became infected after feeding on patients on the seventh day after onset. Nguyen et al. (32) further demonstrated that patients with DENV-1 and DENV-2 infections can still be infectious to mosquitoes up to 2 days after defervescence, albeit this was rare. For patients infected with DENV-3 and DENV-4, viremia had declined below the required infectious dose for mosquitoes to become infected by this time. In addition, Nguyen et al. (32) demonstrated that patients with a high early viremia have a greater probability of having an extended duration of infectiousness. Intuitively, a DENV-infected person with a longer duration of viremia has a greater chance of being bitten by, and infecting, a naïve *Aedes aegypti* mosquito. Therefore, patients with a high early viremia generally have a greater time-window of infectiousness for mosquitoes.

SYMPTOMATIC VS. ASYMPTOMATIC INFECTIONS

Ambulatory patients with symptomatic DENV infections have viremia levels that are unquestionably likely to render them infectious to mosquitoes (32). Individuals who are asymptomatic with a DENV infection also have detectable levels of virus circulating in the blood (38, 39), but the question remains open as to whether or not inapparent DENV infections have high enough viremias to be infectious. Because inapparent DENV infections are common (40, 41), it follows that they could play a role in the maintenance of DENV in its natural transmission cycle, should their viremias be above the infectious threshold level.

UNDERSTANDING THE EXTENT OF DENV TRANSMISSION THAT IS A RESULT OF ASYMPTOMATIC INFECTIONS

While the estimated number of asymptomatic DENV infections (over 290 million cases each year) outweighs that of symptomatic infections around the world (8) the contribution of these asymptomatic infections to the continued transmission of DENV remains to be elucidated. Definitive studies to determine whether acute asymptomatic cases are able to infect susceptible mosquitoes will give insight to the contribution of asymptomatic infections to the overall transmission dynamics. These can be done in two ways. The first involves more detailed surveillance and tracing of contacts of dengue index cases than that done in current longitudinal studies [e.g., those in Thailand (40), Nicaragua (41), and Peru (42)]. Increasing the frequency of blood draws of these contacts will help to identify asymptomatic cases at the earliest possible time. At first

observation of viremia, the individual can be exposed to susceptible mosquitoes that are then tested for infection after a suitable incubation period.

Alternatively, we can gain this same knowledge in human challenge experiments, along with vast amounts of other information, on early infection dynamics (the portion of dengue pathogenesis that is least understood because patients only present to health care professionals after symptoms have already manifested). In human challenge studies, some participants will likely develop asymptomatic infections and the question of whether these individuals are infectious to mosquitoes can be tested in a controlled setting. In addition, such studies should also take such an opportunity to study the early infection dynamics in the participants in human challenge experiments with the aim in investigating the determinants of an infection becoming symptomatic or asymptomatic.

To date, there are few studies that have even demonstrated that asymptomatic infections result in a detectable viremia. Studies in both Nicaragua and Indonesia have described persons with acute asymptomatic DENV-1 and DENV-2 infections (having successfully amplified viral RNA by RT-PCR and/or by directly isolating the virus from the blood draw) using an index-case cluster surveillance approach described above (38, 39). This demonstrates it is indeed possible to study asymptomatic infections within the human host, but unfortunately in both studies, DENV viremia was not quantified, and mosquitoes were not exposed to the blood of these subjects, thus it is unknown if these individuals were infectious. Duong et al. (43) reported the first and only quantification of viremias in asymptomatic cases in the literature, however, these investigators did not assess infectiousness to mosquitoes. Until empirical evidence is obtained that supports the fact that mosquitoes can become infected, and infectious, after directly feeding on asymptomatic DENV infections, one cannot ascertain the extent to which these many millions of clinically silent infections are contributing to ongoing DENV transmission, or whether they are effectively dead-end hosts.

MOSQUITO SUSCEPTIBILITY TO INFECTION

Vector competence (VC) assays of mosquito susceptibility to DENV frequently test some combination of mosquito infection, dissemination, and onward transmission of virus. One factor potentially influencing our estimates of VC is that many studies have used artificially derived infectious blood meals to orally infect mosquitoes. In the first half of the twentieth century, mosquitoes were routinely fed on people suffering from dengue (33, 34, 36, 37, 44–48). When the weight of DENV research began to take place in non-endemic countries, a need for alternative methods arose. Since then, ordinarily, studies infect mosquitoes using artificial blood meals, consisting of a non-human blood source (often being derived from rabbit or pig), spiked with infectious virus grown in cell culture. While there are benefits of feeding mosquitoes using artificial blood meals (e.g., larger numbers of mosquitoes can be used, viral titers within the blood meal can be manipulated), employing the natural transmission mode to infect mosquitoes will help better describe the three-way human–mosquito–virus relationship in nature. Recognized factors influencing the VC of *Aedes aegypti* for DENV transmission are described below.

RELATIVE VECTOR COMPETENCE OF *AEDES AEGYPTI* AND *AEDES ALBOPICTUS*

Although *Aedes aegypti* are generally considered to be the primary vectors of DENV, *Aedes albopictus* have been implicated as the primary, if not the sole vector of DENV during some disease outbreaks (49, 50). Empirical studies show the two species do not differ in the competence to transmit DENV; both *Aedes aegypti* and *Aedes albopictus* collected from multiple sites within Cameroon showed no overall difference in their disseminated infection rate to DENV-2 (the same held true for infection with chikungunya virus also) (50). Similar results failing to identify differences in competence between the two species were reported for mosquitoes from the Florida Keys challenged with DENV-1 (51). Although both of these studies used artificial blood meals when infecting the mosquitoes and obtained similar results, the relative competence of these species after feeding on the viremic blood of a dengue case is unknown. A meta-analysis of 14 studies on the relative susceptibility of *Aedes albopictus* and *Aedes aegypti* suggests that *Aedes albopictus* are more susceptible to midgut infections than *Aedes aegypti*; however, the ability of the virus to disseminate in the latter mosquito is greater, suggesting a greater potential for transmission in nature (52).

VIRUS CONCENTRATION IN THE BLOOD MEAL AND THE EXTRINSIC INCUBATION PERIOD

Numerous studies demonstrate that the proportion of mosquitoes that become infected with DENV depends on the concentration of virus in the blood meal (32, 53). Bennett et al. (53) identified a positive association between viral titer of DENV-2 in the infectious blood meal and the proportion of *Aedes aegypti* with an infected midgut. Once infected, however, rates of dissemination in the same mosquitoes showed no such association. As described in more detail above, in more than 200 patients with naturally acquired DENV infections, Nguyen et al. (32) detected a positive correlation between mosquito infection prevalence and the titer of virus in human blood (**Figure 1**), consistent across all four serotypes.

Viral titer can also influence the time that it takes for a mosquito to become infectious. Watts et al. (54) demonstrated that infecting Thai *Aedes aegypti* with a low titer of virus resulted in an extended period (up to 25 days) before the mosquitoes were able to transmit DENV-2 to naïve rhesus monkeys, compared to when using a higher titer of virus, where it took only 12 days after incubation at the same holding temperature of 30°C.

ENVIRONMENTAL TEMPERATURE

Environmental temperature has long been implicated in altering mosquito VC to transmit viruses. A positive correlation between mean exposure temperature and the proportion of mosquitoes that become infected with the virus exists, that is bound by upper and lower limits (54–56). The lower the temperature, the longer it takes for the virus to replicate to high enough concentrations to be transmissible (and be detectable using laboratory techniques), but at high temperatures virus replication rates are greater, and the minimum time for the mosquito to complete the extrinsic incubation is decreased. Some populations differ in these values,

but estimates for minimum and maximum thresholds for transmission (i.e., the temperature at which a mosquito can become infectious) at constant temperatures are around 13°C at the lower end (55), and 35°C at the upper end (54, 56) for *Aedes aegypti* mosquitoes (although higher temperatures are not known to have been tested). It is not known what these upper and lower limits are for *Aedes albopictus*.

Testing of the upper thresholds proves difficult, because after mosquitoes have been reared at such high temperatures (*cf.* 38°C and above) the lifespan of the mosquito is reduced due to the negative effect of the high heat; their flight activity is almost negligible and they are unable to source a blood meal (57). Therefore, assessing VC at such high temperatures must be performed at least in semi-unnatural conditions (offering the blood meal to the mosquitoes while at a cooler temperature and then placing them back at the exposure temperatures).

Several recent VC studies investigating transmission of mosquito-borne pathogens have also shown that using natural temperature exposures (ones that fluctuate throughout the day, as a mosquito experiences in nature as opposed to constant temperatures) can alter the expected VC of a mosquito population (56, 58–60). Reaction norms for VC (and other life-history traits) as characterized under constant temperatures failed to accurately predict the competence of *Aedes aegypti* for DENV transmission, after exposure to the same mean temperature, but with the addition of daily temperature fluctuations. Large fluctuations in the order of ~19°C around a low mean temperature of 20°C were shown to increase the number of *Aedes aegypti* that became infected with DENV-1, and accelerated the time that it took for dissemination to occur (by around 10 days) (56). Conversely, around a mean temperature of 26°C, one that is commonly used for laboratory-based experiments, the same magnitude of fluctuations had the opposite effect; there were fewer mosquitoes that developed a disseminated infection, and the first time dissemination observed was extended by 4 days (60). These studies highlight that it is important to empirically test mosquitoes under conditions representative of their natural environment to accurately measure VC used for modeling purposes.

Humidity changes may also play a role in mosquito VC, but precise measurements under variable humidity regimes have not been made. It is known that desiccation under dry conditions can place mosquitoes under stress. This stress may exacerbate the inability of the mosquito to fight off a viral infection, or indeed, may negatively impact the virus, because the mosquito may utilize available cellular resources for their maintenance before the virus has the opportunity to use them. At least in *Aedes albopictus*, changes in humidity can enhance the effect of changes in temperature affecting mosquito fecundity (61), and it follows that this is surmised to be the same in the closely related *Aedes aegypti*. More in-depth studies are required to elucidate the effect of humidity on VC indices.

POPULATION EFFECTS, AND INTERACTIONS BETWEEN VIRAL AND MOSQUITO GENOTYPES

Populations of mosquitoes reportedly vary in their susceptibility to DENV infection (53, 62, 63), which can alter the accuracy of predictions of transmission dynamics among populations. On a large geographic scale, Gubler et al. (62) demonstrated population-specific differences in the ability of mosquitoes to become infected with each of the four DENV serotypes. Between populations, there were consistent patterns of high and low infection when exposed to each of the serotypes, suggesting that the factors controlling infection by each of the DENV serotypes is uniform and possibly conserved. Even on a relatively small scale, Bennett et al. (53) found that there was significant variation in the ability of 24 populations of mosquitoes from Mexico and USA to become infected with a DENV-2 strain.

There is also the suggestion that within a single population of mosquitoes, susceptibility to infection by different viral isolates/genotypes may vary (64–66). After challenging three isofemale lines of *Aedes aegypti* that were derived from Ratchaburi, Thailand, with three Thai isolates of DENV-1 virus (that were in current circulation), Lambrechts et al. (64) demonstrated that each of the Ratchaburi isofemale lines were most susceptible to infection by the viral isolate from the same city, Ratchaburi, as opposed to those from Kamphaeng Phet or Bangkok. A follow-on study identified polymorphisms at the *dicer-2* locus as being associated with these phenotypic differences in mosquito VC. Further studies demonstrating that this result holds true for mosquitoes derived from other populations are needed to show the generality of the phenomenon. In any case, the differences demonstrated between mosquito populations in their susceptibility to DENV infections suggest that mathematical models of DENV transmission need to consider the nuances of specific mosquito–virus interactions in their parameterization.

BLOOD-FEEDING BEHAVIOR AND PREFERENCES OF DENV MOSQUITO VECTORS

One of the challenges standing in the way of developing targeted intervention approaches for the mosquito to human transmission cycle include a lack of understanding of mosquito behaviors, including that of host-seeking. A cornerstone of the DENV transmission cycle is the mosquito vector, and without an infected mosquito's success in seeking a suitably DENV-naïve host, transmission would cease and the virus would die. Since other bacteria and viruses manipulate the biology and behavior of their hosts to facilitate their own transmission (67, 68), it is plausible that DENV may do the same. Studies on the blood-feeding behavior of DENV-infected mosquitoes have examined duration of probing and feeding (69, 70), transmission efficiency during probing (71), and motivation and avidity to feed (72). While DENV infections may increase the duration of feeding and the likelihood of re-feeding after interruption (as tested using either mice or guinea pigs), no studies have directly investigated human host-seeking ability.

Hypothetically, if an uninfected mosquito is potentially attracted to human hosts with a high body temperature (e.g., as a result of fever), does DENV then manipulate the physiology of an infected mosquito to be more attracted to people with lower body temperature (e.g., those that are likely to be uninfected) for their subsequent meals, to increase the likelihood of transmission? Can DENV increase the frequency and/or desire to blood feed, leading to mosquito vectors feeding on multiple hosts, thereby enhancing transmission? Finally, does the virus alter the physiology of

the human host (other than causing high fever) in ways that are detectable to a mosquito, making them more attractive? Investigating the host-feeding preferences and host-seeking ability of infected and uninfected mosquitoes can help elucidate the extent to which DENV manipulates its mosquito vectors.

OTHER FACTORS INFLUENCING INFECTION

Mosquitoes have an increased risk of infection when exposed to naturally infected dengue patients when they have a high tympanic temperature and high plasma viremia (32). With the progression of illness in a patient, IgM and IgG titers continue to increase until after viremia declines beyond a detectable limit. With this increasing day of illness and associated IgM and IgG titers, *Aedes aegypti* experience a decreasing risk of DENV infection (32). Increased titers of these antibodies in the blood may directly influence mosquito susceptibility, by neutralizing virus and preventing infection of the midgut.

NOVEL ENTOMOLOGICAL STRATEGIES FOR REDUCING DENV TRANSMISSION IN THE FIELD

A number of novel strategies are being developed that control mosquito populations. These include but are not limited to the use of genetic manipulations of mosquitoes, fungus, and bacteria to curb pathogen transmission. The RIDL (release of insects carrying a dominant lethal) technique releases genetically modified males into a mosquito population that carry a late-acting lethal, development gene that is transmitted to each of its progeny (73). Fungal biopesticides have also been proposed for control of mosquito transmission of pathogens (74).

Another of these strategies intends to release mosquitoes infected with the intracellular bacterium *Wolbachia pipientis* (75, 76). In *Aedes aegypti*, *Wolbachia* manipulate the host reproduction system to enhance its own vertical transmission between generations, but can also reduce host lifespan (77), and critically interfere with DENV replication (78). The level of virus interference in *Aedes aegypti* is however dependent on the bacterial strain.

Releasing mosquitoes into the wild that contain this intracellular bacterium aims to reduce the ability of mosquitoes to transmit DENV under field conditions. After the initial establishment phase of the release, this biological control strategy is self-maintained due to *Wolbachia*'s ability to drive itself into a population of hosts, thereby increasing the benefit of this strategy by decreasing long-term maintenance costs. Additionally, the technology can be implemented relatively cheaply, meaning that countries that face a large dengue burden may see the greatest value in its implementation. There are also multiple strains of the bacteria that can be utilized, with different incompatibility phenotypes, offering the opportunity for multiple releases. Field releases of *Wolbachia*-infected *Aedes aegypti* have already occurred in Northern Australia, Vietnam, and Indonesia with the aim of suppressing DENV transmission.

One theoretical concern about this strategy is the long-term efficacy of the program due to evolutionary changes in the genomes of vector, virus, and/or bacteria. Evolution may erode the viral replication inhibition effect of the bacteria, increased virulence of the virus in humans, and decrease the life-shortening phenotype in the bacterial host, as seen in the native Drosophila host of the life-shortening *Wolbachia* strain (79). An objective discussion of the potential evolutionary changes in the *Wolbachia* vs. DENV relationship, within the human–*Aedes aegypti* framework, is presented by Bull and Turelli (80).

SUMMARY AND RESEARCH PRIORITIES

The successful transmission of DENV from human to mosquitoes is a complex interplay of biotic and abiotic factors. Despite this, DENV transmission occurs on a global scale and continues to be the most prevalent arbovirus infection, with an estimated 390 million infections each year (8). At this point, there are several research priorities that would benefit our understanding of human to mosquito transmission, and subsequently aid research and development into the long-term goal of finding effective tools for DENV prevention (e.g., vaccines, prophylactic or therapeutic use of antivirals, and vector control). These research priorities are:

(1) To what extent do asymptomatic infections contribute to ongoing transmission? What proportion of asymptomatic infections result in mosquitoes being capable of transmission? What is the range of viremia required for transmission to occur?
(2) Can antivirals and/or neutralizing antibodies be administered to dengue patients to reduce the potential for DENV patients to infect naïve mosquitoes? Can antibodies neutralize the virus in the mosquito before it becomes infected? How would this feasibly be administered?
(3) How will dengue vaccines modify viremia after natural exposure? Will they modify viremia to a level that prevents human to mosquito transmission?
(4) What preferences do *Aedes* mosquitoes show toward febrile and non-febrile hosts? Are naïve mosquitoes more attracted to febrile hosts (infected with any arbovirus)? Does DENV manipulate host-seeking behavior in infected mosquitoes?
(5) Can the likely field success of novel dengue control measures, such as *Wolbachia*, be predicted from laboratory studies? Which of the many and complex effects of *Wolbachia* on *Aedes aegypti* life-history traits have the greatest impact on VC?

Advances in our understanding of the DENV transmission cycle in humans and mosquitoes should support the rational development and application of interventions such as vaccines, antivirals, and novel entomological control measures.

AUTHOR CONTRIBUTIONS

Lauren B. Carrington and Cameron P. Simmons conceived and wrote the manuscript.

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Conflict of Interest Statement: Cameron P. Simmons is a consultant to Merck and Sanofi Pasteur for the development of dengue vaccines, and to GSK, Unither Virology, and Tibotec for the development of anti-viral therapies

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Human immune responses to dengue virus infection: lessons learned from prospective cohort studies

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Dengue virus (DENV) continues to spread globally and is a major cause of morbidity and mortality. Currently, there is no antiviral treatment to diminish severe illness or a vaccine to induce protection from infection. An effective dengue vaccine that protects against all four DENV serotypes is a high priority for endemic countries and several candidates are in development by various United States Federal Agencies and private pharmaceutical companies. Challenges faced by dengue vaccine developers include creating tetravalent formulations that provide tetravalent protection, the lack of a correlate of protective immunity, a changing viral landscape as DENV evolves, and a complex viral-host pathogenesis that can result in a spectrum of illness from subclinical infection to severe hemorrhagic fever. There have been a number of long-term prospective studies on DENV transmission and dengue severity that have provided invaluable information on DENV epidemiology and pathogenesis of this disease. In this section, we will review the critical lessons learned from these studies and their application for dengue vaccine development.

Keywords: dengue virus, prospective cohort studies, lessons, vaccine, development

INTRODUCTION

The global dengue pandemic and its associated morbidity and mortality have been covered in other excellent reviews and sections of this textbook and will not be reviewed here. Prospective studies have been a valuable tool in understanding the epidemiology and pathogenesis of dengue virus (DENV) infection. For the dengue vaccine developer specifically these studies offer the advantage of determining the true incidence of infection, the full spectrum of clinical outcomes from subclinical to severe hospitalized illness, risk factors for disease severity, and viral information on the genetics and evolution of DENV and its spatial and temporal spread. Recently, the results of a Phase 2b candidate tetravalent DENV vaccine [yellow fever (YF)-dengue chimeric, Sanofi Pasteur] were published (1). This was conducted in a highly flavivirus antibody experienced cohort of children in Thailand and demonstrated an excellent safety and neutralizing antibody immunogenicity profile. The vaccine however, failed to achieve protection against all four DENV types with an overall efficacy of 30.2%. Efficacy varied by DENV type with the lowest noted against DENV-2 (9.2%), which was the predominant circulating DENV serotype at the time of the trial. The results of this efficacy trial highlighted several development challenges for a dengue vaccine including the lack of a correlation of protection despite the detection of serotype-specific neutralizing antibody.

In this section, the lessons learned from the prospective cohort studies and their application to DENV vaccine development will be reviewed. Though much information has been learned from these important studies, there are six important lessons learned that I believe are critical for DENV vaccines and include: (1) incidence rates will support Phase III efficacy studies though a high degree of temporal and spatial diversity in incidence occurs requiring consideration in choosing populations; (2) demonstration that pre-existing serotype-specific neutralizing antibody does not protect against infection suggests that our current assays to measure neutralizing antibody are not a correlate for protection; (3) the symptomatic to inapparent (S:I) ratio is an epidemiologic correlate for heterologous protective immunity or enhancement and may be a correlate for vaccine efficacy; (4) the time from last infection determines the S:I ratio and provides evidence of a half-life of heterologous protective immunity, which may affect the observed efficacy in DENV vaccines depending on the time point efficacy is measured; (5) there is a high degree of temporal and spatial genetic diversity in the DENVs reinforcing the need for a tetravalent DENV vaccine but also consideration for the need of genotypic-specific protection as well; (6) changing population dynamics and viral evolution with older individuals becoming infected suggest that immunosenescence and potential viral escape mutants should be considered when formulating DENV vaccines.

Abbreviations: DENV, dengue virus; DF, dengue fever; DHF, dengue hemorrhagic fever; NAb, neutralizing antibody; PRNT, plaque reduction neutralizing titer.

PROSPECTIVE COHORT STUDIES

Prospective cohort studies on DENV transmission; the surveillance of a select group of individuals over time for DENV infection, are an invaluable source of information on the incidence of infection, viral, and host factors that lead to subclinical infection or severe disease, and understanding the full burden of DENV infection. For testing DENV vaccines, they are essential in determining the true efficacy of a vaccine. **Table 1** is a summary of the published prospective dengue cohort studies to date. The first prospective cohort study was conducted in Rayong, Thailand in January 1980 among children who were sampled from schools

Table 1 | Summary of prospective cohort studies of DENV transmission and disease.

Study site	Population size[a]	Age range (years)	Study period	Incidence (average)				
				Dengue infection (%)	Symptomatic dengue	Hospitalized dengue	Severe dengue	Symptomatic: asymptomatic ratio
Rayong, Thailand (2)	1,056	4–14	1980–1981	39.4	n/a[b]	0.7%	0.7%	n/a
Bangkok, Thailand (3)	1,757	4–16	1980–1981	11.8	0.7%	0.4%	0.4%	1:8
Yangon, Myanmar (4)	12,489	1–9	1984–1988	5.1	n/a	0.3%	0.2%	n/a
Yogyakarta, Indonesia (5)	1,837	4–9	1995–1996	29.2	0.6%	0.4%	0.4%	n/a
Kamphaeng Phet I, Thailand (6)	2,119	7–11	1998–2002	7.3	3.9%	1.0%	0.6%	1:0.9
Iquitos, Peru (7)	2,300	5–20	1999–2005	34.5	n/a	n/a	n/a	n/a
West Java, Indonesia (8)	2,536	18–66	2000–2002	7.4	1.8%	0.1%	0.1%	1:3
Managua, Nicaragua (9, 10)	1,186	4–16	2001–2002	9.0	0.85%	n/a	n/a	1:13–1:6
Maracay, Venezuela (11)	981	5–13	2001–2002	16.9	n/a	n/a	n/a	n/a
Kamphaeng Phet II, Thailand (12)	2,095	4–16	2004–2006	6.7	2.2%	0.5%	0.1%	1:3.0
Ratchaburi, Thailand (13, 14)	3,015	3–11	2006–2009	3.6	3.6%	1.6%	0.3%	n/a
Managua, Nicaragua (10)	3,800	2–9	2004–2010	9.0	0.85%	n/a	n/a	1:13–1:6
Long Xuyen, Vietnam (15)	2,190	2–15	2004–2007	3.0	3.0%	1.2%	1.2%	n/a
Southeast Asia (Indonesia, Malaysia, Philippines, Thailand, and Vietnam (16)	1,500	2–14	2010–2011	11.4	n/a	n/a	n/a	n/a

[a] *Number in cohort tested for dengue antibody (incidence denominator).*

[b] *n/a, not available; not provided in the published paper.*

and households (2). Pre- and post-epidemic cohort blood samples determined that the incidence of dengue infection in 251 seronegative children was 39.4%. The average incidence of hospitalized dengue was 0.7%. Of the shock syndrome cases admitted to the hospital, the major risk factor for severe infection was secondary infection. The Rayong study was the first to establish the high burden of dengue illness in Thailand, the association of secondary dengue infections with severe dengue illness, the circulation of all four DENV serotypes and the preponderance of one specific serotype in a given epidemic year, and the importance of sequential dengue serotypes in producing shock syndrome. Despite the variation in incidence amongst the prospective studies listed in **Table 1**, the incidence of severe hospitalized disease is remarkable consistent, all countries where they were conducted have a high burden of DENV infection and >80% of severe infections are associated with subsequent DENV infections after a primary infection.

The second prospective study was a 2-year (1980–1981) school-based study involving 1,757 children, ages 4–16 years, in Bangkok, Thailand (3). This was the first study to use active surveillance using school absence as an indicator of potential illness. Antibody titer revealed that 50% of the enrolled students had evidence of dengue antibody, likely indicative of a DENV infection experienced prior to the age of 7 years, and the first to demonstrate that even at this young age the population is already highly flavivirus antibody experienced. Most (87%) of the students who became infected during the study period were asymptomatic as determined by lack of clinical illness. Important study findings were an incidence in dengue-naïve participants of 6.3%, in dengue-experienced participants an incidence of 5.5% and among symptomatic infected children a hospitalization rate of 53%. The symptomatic-to-asymptomatic ratio of DENV infection was 1:8. This study confirmed the importance of subsequent DENV infection as a risk factor for severe infection with an odds ratio for developing DHF in participants with pre-existing dengue immunity ≥6.5. The importance of this prospective study was as the first study to determine the full burden of DENV infection within a cohort, the incidence of infection and the relationship of pre-existing DENV immunity to dengue disease severity.

In the last decade a number of prospective cohort studies on DENV transmission have been performed adding to our knowledge on DENV incidence and risk factors for severe infection. These include an ongoing study in Iquitos, Peru (the first in the Americas), one in Indonesia (the first in adults), an ongoing study in Nicaragua, studies in Venezuela, Vietnam, Malaysia, Philippines, Vietnam, and ongoing studies in Kamphaeng Phet, Thailand.

INCIDENCE RATES WILL SUPPORT PHASE III EFFICACY STUDIES THOUGH A HIGH DEGREE OF TEMPORAL AND SPATIAL DIVERSITY IN INCIDENCE OCCURS REQUIRING CONSIDERATION IN CHOOSING POPULATIONS

The prospective studies from both the America and Asia have demonstrated a high burden of DENV infection and incidence rates that clearly support economically feasible DENV vaccine efficacy studies. With an average incidence of symptomatic DENV infection of 2%, a sample size calculation using an 80% efficacy of the vaccine, an alpha value of 0.05, power of 0.8, will require a total sample size of vaccinated and controls of 1,452 volunteers.

Studies in Iquitos, Peru and in Kamphaeng Phet, Thailand (referenced in **Table 1**) demonstrated the heterogeneity of DENV incidence and serotype-specific transmission spatially and temporally and illustrated elegantly in schools in Kamphaeng Phet, Thailand. In Kamphaeng Phet, dengue incidence and serotype-specific transmission was cyclical in each school, with relatively mild years followed by more severe years. A proportion of schools had a severe dengue year while other schools a short distance away had less severe dengue. Similarly, one school would have a DENV-2 outbreak and another a short distance away a DENV-3 outbreak. The diversity of incidence both spatially and temporally is an important observation from these studies in designing dengue vaccine efficacy studies and in estimating and choosing the population and geographic location required to assess statistical efficacy.

PRE-EXISTING SEROTYPE-SPECIFIC NEUTRALIZING ANTIBODY DOES NOT PROTECT AGAINST INFECTION SUGGESTS THAT OUR CURRENT ASSAYS TO MEASURE NEUTRALIZING ANTIBODY IS NOT A CORRELATE FOR PROTECTION

Historically and currently, DENV neutralizing antibody has been a serologic biomarker to measure previous and current DENV serotype-specific infection (1, 17, 18). The role of homotypic and heterotypic antibody as a marker of immunity was first described in studies performed by Sabin in the 1940s in human volunteers (19, 20). The concept as demonstrated was that following a serotype-specific DENV infection, homotypic antibody develops to that specific serotype that last life-long and becomes a biomarker that indicates previous infection to that serotype and thus durable immunity. Heterotypic, cross-reactive antibody to other DENV serotypes following a serotype-specific infection also develops, is transient, and may modify disease severity when infected with another serotype. The development of the plaque reduction neutralization titer (PRNT) assay and its variation for DENV became a relatively high-throughput assay to measure this biomarker for previous and current serotype-specific protection (17). As such it has evolved to be an important biomarker to measure DENV vaccine performance in pre-clinical and clinical DENV vaccine trials. We have demonstrated that this assay has a high degree of variability in its performance depending on the cell lines and prototype viruses used and the detection of heterotypic neutralizing antibody creates confusion in its interpretation of whether an individual is protected or not from a specific serotype (21). In the Kamphaeng Phet cohort studies, the active surveillance for infection and analysis of archived sera from infected individuals allowed the examination of serotype-specific PRNT antibody just prior to severe DENV infection (22). What was demonstrated was the observation that individuals hospitalized for severe serotype-specific DENV had detectable PRNT titers to that serotype using either prototype DENV and to their own isolated virus approximately 6 months prior to infection. The implication of this finding was that the PRNT was not an adequate biomarker for serotype-specific protection. Ten years later this finding was reiterated during the vaccine efficacy studies of the ChimeriVax vaccine (1). Despite high levels of DENV-2 neutralizing antibody as detected by PRNT, only 9.2% were protected. These findings highlight the need for the development of biomarkers that are highly predictive of serotype-specific DENV protection and currently a major obstacle in the development of DENV vaccines.

SYMPTOMATIC TO INAPPARENT RATIO IS AN EPIDEMIOLOGIC CORRELATE FOR HETEROLOGOUS PROTECTIVE ANTIBODY AND MAY BE A CORRELATE FOR VACCINE EFFICACY

The original Bangkok prospective study demonstrated the full burden of DENV infection, documented the occurrence of subclinical infection and introduced the concept of the S:I ratio (3). In the Kamphaeng Pet cohort studies, we have demonstrated that the S:I ratio is not a fixed variable but there is a variance in the ratio that varies spatially and temporally (6). This ratio was fluid with schools experiencing more symptomatic disease, high S:I ratio, than other schools which shifted the following year where schools had more subclinical infections, lower S:I ratios, while other schools experienced high S:I ratios. This variation in the S:I ratio when analyzed closely is not a random event but occurs in a cyclic pattern with one school having a high S:I ratio, more symptomatic infection, followed in the same school by years of lower S:I ratios, less symptomatic infection. This pattern was reflected at the individual school level and also as a population as a whole. An explanation of this variance is that the S:I ratio is an epidemiologic correlate for an undercurrent of heterotypic protective immunity that may not prevent infection but may modify disease severity. At an individual level what this implies is that following a serotype-specific infection there is the generation of heterotypic antibody that modulates severity of infection that fades with time. At the same time there is a heterotypic enhancing antibody that can influence the production of more severe dengue infection. Expanding from an individual level, this effect applies to local populations such as schools, villages and to the population as a whole and will be discussed in more detail in the next section. The data suggest that the S:I ratio is an epidemiologic correlate for this protective or disease enhancing heterotypic antibody and reflects the time-dependant flavivirus experience of a population. For DENV vaccine studies, the S:I ratio may be a very important correlate of the vaccine's ability to confer heterotypic protection or enhancement of disease severity and potentially an important efficacy correlate to follow over time in a population.

TIME FROM LAST INFECTION DETERMINES THE S:I RATIO AND PROVIDES EVIDENCE OF A HALF-LIFE OF HETEROLOGOUS PROTECTIVE IMMUNITY WHICH MAY AFFECT THE OBSERVED EFFICACY IN DENV VACCINES DEPENDING ON THE TIME POINT EFFICACY IS MEASURED

As discussed, the S:I ratio in a population over time is an epidemiologic correlate that reflects an undercurrent of both heterotypic protective and enhancing immunity that is time-dependant. We and others have demonstrated that the time from last infection determines the S:I ratio and that there is a measurable half-life of heterologous protective immunity (23, 24). This was elegantly demonstrated in the Kamphaeng Phet cohort studies and recently published (23). Data from the prospective cohort studies were analyzed for subclinical and symptomatic DENV infections in schoolchildren from 1998 to 2007. Children who experienced one

or more DENV infection were selected as the population for analysis (2,169 person-years of follow-up). Demonstrated from the analysis was a shorter time interval between infections was associated with subclinical infection in children who were seronegative for DENV at enrollment; seronegative being an important pre-existing condition to document primary to secondary infections as compared to the more complicated second to third infections. This time interval was an average of 1.6 years from first infection that resulted in a subclinical infection, 1.9 years for symptomatic dengue fever (DF), and 2.6 years for the onset of severe hospitalized dengue hemorrhagic fever (DHF). These findings indicate that there is a window of cross-protection following DENV infection that last approximately 1.6 years following infection. The implications of these findings for DENV vaccine efficacy studies are subtle but important. The goal for a tetravalent DENV vaccine is to confer durable tetravalent DENV immunity and protection against severe illness. If one serotype in the DENV tetravalent formulation in particular induces a brisk immune response, the results from the prospective cohort studies would suggest that there will be heterotypic protective immunity from that one serotype to other serotypes conferring tetravalent protection that lasts for 1.6 years. If true, then measuring efficacy at 1 year following vaccination as the most recent DENV vaccine trial did, would not be a true measurement of tetravalent protection. A more appropriate efficacy time point would be at least 3 years following vaccination as a true measurement of efficacy. This of course would mean a longer duration of observation of the population following vaccination and cost of the study.

THERE IS A HIGH DEGREE OF TEMPORAL AND SPATIAL GENETIC DIVERSITY IN THE DENVs REINFORCING THE NEED FOR A TETRAVALENT DENV VACCINE BUT ALSO CONSIDERATION FOR THE NEED OF GENOTYPIC-SPECIFIC PROTECTION AS WELL

It is thought that the DENV transmission evolved into four distinct serotypes approximately 1,000 years ago and each of these four serotypes emerged into a cycle of transmission between humans and its mosquito vector approximately 125–320 years ago (25, 26). Phylogenetic analysis suggests that the DENVs are rapidly evolving with major clade replacements and genetic shifts occurring in populations endemic for DENV (25–28). Asia has been pivotal in the evolution of DENV as the location of the first cases of the more severe form of DENV infection, DHF, which made its first appearance in the 1950s first in the Philippines then in Thailand (29). The current Asian genotypes of each serotype are considered more severe, result in more severe dengue illness, than the American genotypes (30). Evidence suggests that DENV circulation in Asia due to its population growth and urbanization, high vector burden, and high level of pre-existing flavivirus seroprevalence, contribute to the increase in genetic diversity which is estimated as increasing at a factor between 14 and 20 in the last 30 years (31). The overall impression of DENV evolution in Asia is the active transmission of viruses in individuals who are highly flavivirus antibody experienced causing evolutionary pressure on the virus to evolve to escape and utilize pre-existing flavivirus immunity. By its nature the current evolving DENVs are adept at escaping heterologous neutralizing antibody and using it as a means to attain high viral load levels and more severe disease through antibody enhancement.

To understand the evolution and phylogeography of DENV, the prospective cohort studies in Kamphaeng Phet have been isolating and sequencing DENVs from individuals in specific geographic areas and over time. In ongoing studies, viruses are isolated and through the use of global positioning system (GPS) the exact geographic position located allowing specific spatial and temporal analysis. Analysis of isolated viruses demonstrated diverse viral genetic variation in both time and space in the Kamphaeng Phet population with multiple viral lineages circulating within individual schools and villages (32). This implies that there is frequent gene flow of DENV into this microenvironment as individuals move from major urban areas into smaller village. At the microenvironment level, there was clustering of specific viral genotypes within individual schools that are highly conserved from year to year and evidence of frequent viral gene flow among schools closely related in space and time. These results suggest that there is a combination of frequent viral migration into Kamphaeng Phet coupled with population subdivisions of conserved viruses that shape the genetic diversity of DENV at a local and population scale. Taken together is a picture of a virus that is evolving rapidly, spread by population movement, and due to the restricted flight of the vector, genetically conserved at a microenvironment level.

For a DENV vaccine these findings have many implications with the most important is that the current vaccines using historical prototype viruses collected in the 1980s may no longer provide the protection to current circulating DENV genotypes and raises the question if protection needs to be both serotype and genotype specific. This question was raised during the ChimeriVax vaccine trial where the DENV-2 vaccine strain did not match the circulating wild-type DENV-2 and failed to produce protection (1).

CHANGING POPULATION DYNAMICS AND VIRAL EVOLUTION WITH OLDER INDIVIDUALS BECOMING INFECTED SUGGEST THAT IMMUNOSENESCENCE AND POTENTIAL VIRAL ESCAPE MUTANTS SHOULD BE CONSIDERED WHEN FORMULATING DENV VACCINES

Dengue illness in endemic countries is considered to be primarily a pediatric disease due to the high degree of transmission and infection that occurs at a young age. Adults are considered highly flavivirus experienced and thus protected from subsequent dengue illness. For this reason, the prospective cohort studies have largely been performed in pediatric populations and illness criteria of severity of disease developed in pediatric populations. In our own cohort studies, we have noted that the age of symptomatic DENV infection has been increasing slowly over the last decade by a median increase of 2–4 years. This has been reflected in Thailand as a whole and interestingly in the rest of Southeast Asia (33). An analysis and mathematical modeling of this phenomenon was performed on data from each of the 72 provinces of Thailand (33). What was described was that the force of infection has declined by 2% each year with the strongest predictor of this change the median age of the population, which was reflective of the changing demographics of the population. The authors' conclusion was that lower birth and death rates decrease the flow of susceptible individuals into the population and thereby increase

the longevity of immune individuals. For a DENV vaccine, the implications are despite the decrease in the force of infection the critical vaccination fraction has not changed declining from 85 to 80% (2). A key point in this argument however is the longevity of immune individuals and if protection is life-long. Recently, in our own studies in Kamphaeng Phet we have been documenting a large number of adults who are older than 50 years of age with one 80-year old hospitalized with severe DENV infection. These are individuals who have never moved from Kamphaeng Phet, a highly endemic region for DENV, and defy statistical probability of never having been infected by one or more DENVs in their previous experience. This is under active investigation but does suggest that the aging immune system, immunosenescence, might be a factor in older individuals becoming ill from DENV infection or, and particularly worrisome, that the current strains/genotypes have evolved as escape mutants and individuals are no longer protected from current wild-type DENV strains/genotypes despite infection from another genotype but same serotype in the past. This as noted in the previous section has implications on the current DENV vaccine candidates and whether their vaccine prototype strains/genotypes are applicable to current circulating genotypes.

SUMMARY

The prospective studies have been invaluable in increasing our understanding on the epidemiology and pathogenesis of this globally important virus and disease. Discussed are important lessons learned from these studies that have direct application to current DENV vaccine development and testing as they move forward in their regulatory development to a licensed vaccine. Identified are critical issues that are challenges for the development of an effective tetravalent DENV vaccine. Additional research is needed to address these areas that are critical to vaccine testing and evaluation. Expanding cohort studies to include those countries where dengue is inadequately characterized is critical for us to further understand the unique viral and host factors that contribute to differences in dengue risk and vaccine response.

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T-cell immunity to infection with dengue virus in humans

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Dengue virus (DENV) is the etiologic agent of dengue fever, the most significant mosquito-borne viral disease in humans. Up to 400 million DENV infections occur every year, and severity can range from asymptomatic to an acute self-limiting febrile illness. In a small proportion of patients, the disease can exacerbate and progress to dengue hemorrhagic fever and/or dengue shock syndrome, characterized by severe vascular leakage, thrombocytopenia, and hemorrhagic manifestations. A unique challenge in vaccine development against DENV is the high degree of sequence variation, characteristically associated with RNA viruses. This is of particular relevance in the case of DENV since infection with one DENV serotype (primary infection) presumably affords life-long serotype-specific immunity but only partial and temporary immunity to other serotypes in secondary infection settings. The role of T cells in DENV infection and subsequent disease manifestations is not fully understood. According to the original antigenic sin theory, skewing of T-cell responses induced by primary infection with one serotype causes less effective response upon secondary infection with a different serotype, predisposing to severe disease. Our recent study has suggested an HLA-linked protective role for T cells. Herein, we will discuss the role of T cells in protection and pathogenesis from severe disease as well as the implications for vaccine design.

Keywords: DENV, T cells, protection, pathogenesis, HLA, vaccines

WORLD WIDE MEDICAL AND SOCIETAL SIGNIFICANCE OF DENV AND DENV INFECTION

Dengue virus (DENV) is the causative agent of dengue fever, the most prevalent mosquito-borne viral illness in humans and is primarily transmitted by the mosquitoes *Aedes aegypti* and *Aedes albopictus* (1). The world wide distribution of these two major vectors puts nearly a third of the global human population at risk of infection (2). It was recently reported that as many as 390 million dengue infections occur worldwide each year, thus making this infection potentially even more prevalent than malaria (3). Recent outbreaks in Europe (4) and the US (5, 6), led to the recognition of DENV as a Category A priority pathogen by NIAID and the classification of DENV as a domestic re-emerging disease threat by the CDC.

Disease can be induced by any of the four serotypes of DENV (DENV1–4). DENV-associated disease in most cases ranges from asymptomatic to an acute self-limiting febrile illness. However, in a small proportion of patients, the disease can exacerbate and progress to severe forms of dengue disease [dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS)], characterized by severe vascular leakage, thrombocytopenia, and hemorrhagic manifestations (7). Infection with one DENV serotype presumably results into life-long immunity to the infecting serotype but does only confer short-term protection against the other serotypes (8). In fact, the severe forms of DENV disease are more often observed in individuals experiencing a secondary infection with a different serotype (9, 10). Besides prior infection with one serotype, other factors influencing the disease outcome are the strain of infecting virus, age and gender, nutritional status, and the genetic background of the patient (11–16). No licensed vaccine or effective anti-viral therapy is currently available and treatment is largely supportive in nature, thus increasing the economic and disease burden on public health systems in tropical and subtropical countries around the globe (17–19).

DENV-SPECIFIC T-CELL RESPONSES

T-CELL EPITOPE IDENTIFICATION

A previous analysis (20) documented the substantial knowledge gaps existing at the level of defined T- and B-cell immune responses. Over the last years, the situation has improved considerably. As of June 2013, the immune epitope database (IEDB)[1] lists 369 antigenic regions identified in humans and 71 identified in HLA transgenic mice. It has been shown that $CD8^+$ T cells most vigorously and frequently recognized the NS3, NS4B, and NS5 proteins, whereas the capsid, envelope, and NS3 proteins are the dominant targets for $CD4^+$ T cells (21–25). In parallel, but beyond the scope of this review, significant strides have been made in the definition of DENV-related B cell epitopes.

Despite these efforts, significant gaps yet remain. First, the vast majority of DENV epitopes described in the literature and reported in the IEDB are restricted by HLA MHC class I alleles, and only 13% of the epitopes are restricted by HLA class II and recognized by $CD4^+$ T cells. Of those epitopes, only a few have defined allele and loci restrictions and characterized functional and phenotypic features of the associated T cells. Thus, a comprehensive analysis of MHC class II restricted responses across all loci is needed in the general population from endemic areas and

[1] www.iedb.org

in patient populations associated with different severity of disease (acute DF versus DHF/DSS). Furthermore, the epitopes recognized after vaccination with experimental vaccines have not been systematically identified or validated. This hampers progress in the field, as the role of MHC class I and class II restricted responses in disease protection and immunopathology cannot be broadly evaluated, and the performance of different vaccines in terms of induction of immune responses in human vaccines remains undetermined.

IMPLICATIONS FOR HLA POLYMORPHISM

T cells recognize a complex of a particular pathogen-derived epitope presented by a specific MHC molecule. Thus, a given epitope will elicit a response in individuals that express MHC molecules capable of binding that particular epitope. MHC molecules are extremely polymorphic, with several thousand variants known in humans (26, 27). Each variant is present with variable frequency, depending on ethnic lineage and geographic locality. As a result, for basic investigations, diagnostic or vaccine applications and to ensure high and non-ethnically biased coverage of different patient populations, it is necessary that the alleles investigated are carefully selected. This is accomplished by selecting those alleles that are most frequent in the various population groups worldwide.

To address this challenge in the context of HLA class I, we have applied a selection process focused on the 27 most common HLA A and B alleles in the general population (25). As previously described, these 27 alleles allowed us to cover at least three out of four possible HLA A and B alleles expressed per donor in 90% of a cohort from the general population of Colombo, Sri Lanka. In the case of HLA class II, we have recently reported the selection of a panel of 27 different allelic variants that affords high coverage of all four HLA class II loci (DRB1, DRB3/4/5, DQ, and DP) (27). Based on publically available population frequency data (28), this panel of HLA DR, DQ, and DP specificities should allow to cover over 98% of individuals in the general population. Notably, the actual coverage achieved by this panel was similarly high in cohorts of distinctly different ethnic composition that we have previously utilized for our studies (29, 30). For each of these class II molecules, we have established quantitative binding assays (27) and generated a sufficiently large number of measurements to enable derivation of quantitative algorithms for predicting binding capacity (27, 31). Predictive algorithms for the most common HLA class I and class II alleles are now publicly available at several web sites, including the IEDB[2]. Additionally, for each molecule we have produced cell lines transfected with a single HLA class II allele that will be useful for fine mapping of HLA restrictions (30). These approaches now represent efficient and valuable tools for epitope identification, especially in the context of large and complex targets.

THE HYPOTHESIS OF ORIGINAL ANTIGENIC SIN AS IT IS RELATED TO A POTENTIAL ROLE OF T CELLS IN DENV PATHOGENESIS

It has been proposed that cross-reactive T cells raised against the original infecting serotype dominate during a secondary heterologous infection, a phenomenon that has been termed "original antigenic sin" (32, 33). This term was first applied to the humoral response to influenza epidemics (34), but has also been observed in $CD8^+$ T-cell responses against lymphocytic choriomeningitis virus (LCMV) (35). This hypothesis postulates that during secondary infection, expansion of pre-existing lower avidity cross-reactive memory T cells dominate the responses over that of naïve T cells that are of higher avidity for the new DENV serotype. It is further hypothesized that peptide variants derived from the secondary infection serotype can induce a response that is qualitatively different from the response induced by the original antigen, such as inducing a different pattern of cytokine production. Variants associated with this phenotype are often collectively referred to as altered peptide ligands (APLs) (36). It is hypothesized that these altered T-cell responses serotype may contribute to a "cytokine storm" during heterologous secondary infection and thus contribute to immunopathogenesis of severe dengue disease (33). However, this hypothesis is in conflict with the observation that heterologous T-cell responses are not always needed to produce severe disease in infants. DHF or DSS in infants generally occurs between the ages of 6 and 12 months in endemic areas (37). When the maternal antibody titer to DENV decreases below a protective level, infants are actually at an increased risk for the development of severe disease despite the fact that they have never been infected with DENV and lack DENV-specific T-cell memory (38). Furthermore, a recent study has shown a temporal mismatch between the $CD8^+$ T-cell response and commencement of capillary leakage, suggesting that $CD8^+$ T cells are not responsible for early triggering of capillary leakage in children with DHF (39).

We have previously reported that "original antigenic sin" is indeed detectable at the level of $CD8^+$ T-cell responses in the general population (25). However, a potential limitation of those studies was that they were conducted at the level of the general population from an endemic area (i.e., Sri Lanka), and did not measure HLA class II restricted epitopes. Furthermore, it is not known whether the studies could capture *in vivo* impaired or altered T-cell responses during acute infection.

LOW MAGNITUDE T-CELL RESPONSES ARE HLA-LINKED AND ASSOCIATED WITH DISEASE SUSCEPTIBILITY

The results presented above suggest that antigenic sin does not significantly impair the quality of T-cell responses in the general population. However, lower quality responses might be associated with the relatively few individuals experiencing more severe clinical outcomes. Previous studies highlight that certain HLA alleles are associated with either increased or decreased risk of clinical manifestations (14, 40–45). However, these studies did not determine whether increased risk might be associated with a hyperactive T-cell response, or conversely whether a higher T-cell response might be associated with a decreased risk. Correlations of HLA-associated disease susceptibility with T-cell responses found that weak T-cell responses correlated with disease susceptibility (25). A possible explanation for these observations would be that certain alleles and epitopes are associated with higher magnitude responses, which are in turn associated with higher degrees of multi-functionality, and thus might be most beneficial in protecting from disease. A detailed analysis of cytokines produced by DENV-specific T cells revealed that stronger responses are indeed

[2]www.immuneepitope.org

associated with multifunctional T-cell responses. Thus, it might be possible that while T cells have a protective role in general in the HLA-linked, lack of a multifunctional T-cell response might contribute to pathogenesis in certain individuals.

ROLE OF T CELLS IN PROTECTION AGAINST DENV INFECTION

The protective role of T cells during viral infection is well established (46). Generally, $CD8^+$ T cells can control viral infection through several mechanisms, including direct cytotoxicity and production of pro-inflammatory cytokines such as IFN-γ and TNFα. Similarly, $CD4^+$ T cells are thought to control viral infection through multiple mechanisms, including enhancement of B and $CD8^+$ T-cell responses, production of inflammatory and antiviral cytokines, cytotoxicity of viral infected cells, and promotion of memory responses (47).

Several lines of evidence suggest that both $CD4^+$ and $CD8^+$ T cells may contribute to protection against homologous reinfection or heterologous dengue infection. It has been shown that DENV-specific human $CD4^+$ T and $CD8^+$ T cells proliferate, produce IFN-γ, and lyse infected target cells (48–50), suggesting that serotype-specific T cells are activated and functional in humans with primary DENV infection (48, 51). Furthermore, higher frequencies of DENV-specific IFNγ-producing T cells are present in children who subsequently develop subclinical infection, compared with those who develop symptomatic secondary DENV infection (52).

Finally, studies in a murine model of DENV infection demonstrated that both $CD4^+$ and $CD8^+$ T cells contribute to protection against DENV challenge (53–56). In parallel to the evidence in the murine model, studies performed previously demonstrated that HLA alleles associated with increased risk of severe disease are also associated with weak $CD8^+$ T-cell responses, and conversely that strong, multifunctional, T-cell responses correlate with alleles associated with protection from severe disease. These data strongly imply a protective role for $CD8^+$ T cells against severe DENV disease in humans (25).

DENV SEROTYPES AND VACCINE DEVELOPMENT

The dengue serocomplex consists of four serotypes, each of which is made up of several genotypes (57). The four serotypes share 65–75% genetic homology with each other but are antigenically distinct (58). This high degree of sequence variation in a pathogen, characteristically associated with RNA viruses, poses unique challenges to vaccine development. This is of particular relevance in the case of DENV infections because of the more severe disease and immunopathology associated with prior exposure to a different serotype (9). Consequently, the development of DENV vaccines has been hampered by the potential risk of vaccine-related adverse events and the requirement to induce long-lasting protective immune responses against all four DENV serotypes simultaneously. A recent phase 2b proof-of-concept efficacy vaccine trial (59) of a live-attenuated tetravalent chimeric yellow fever-dengue vaccine (CYD23) showed only 30% overall efficacy, demonstrating partial (60–80%) protection toward three of four DENV serotypes. No protection against DENV2 infection was observed, despite three subsequent immunizations and high neutralization titers against all four serotypes.

As reviewed above, T-cell responses have been implicated to have a protective role in DENV infection. Previous data from our lab and others clearly demonstrate that $CD8^+$ T-cell responses dominantly target the non-structural proteins NS3, NS4B, and NS5 (21–25). Since these DENV proteins are absent in the recombinant live-attenuated tetravalent dengue-yellow fever chimeric virus vaccine (60), our results perhaps provide an explanation for the low level of vaccine efficacy observed. Further, our data demonstrate the need to accurately assess T-cell responses (and not only antibody responses) in the context of DENV vaccine development.

Five additional and promising vaccine candidates are being tested in human clinical trials. These vaccines rely on technologies spanning from live-attenuated viruses, vectored vaccines expressing certain dengue proteins, replication-defective vaccines to nucleic acid-based vaccines [reviewed in Ref. (61)]. Since our data raise the possibility that T-cell responses against all DENV proteins might be beneficial or even required for vaccine efficacy, it will be of particular interest to study T-cell epitopes induced by multivalent live-attenuated vaccines and compare them to T-cell responses observed in natural infection.

METHODS TO CHARACTERIZE T-CELL RESPONSES AFTER VACCINATION

Characterization of T-cell epitopes can be performed by a variety of techniques, each associated with distinct advantages and disadvantages. These techniques include: ELISPOT, FACS and ICS assays, cell sorting, and tetramer staining. Though ELISPOT is the most sensitive at detecting low-levels of specific cytokine production, ICS assays are better suited to characterize phenotypes and T cells that are simultaneously producing multiple cytokines. Secretion of particular cytokines such as IFN-γ and TNF-α has been implicated in the induction of DENV-associated immunopathology. IFN-γ has been implicated as having a protective role during DENV infection whereas TNF-α has been implicated as a key mediator of immunopathology (62, 63). Characterization of a broad array of cytokines affords determination of the degree to which the cells responding to a given epitope are polyfunctional effectors. As illustrated in several different systems, T cells with a polyfunctional phenotype capable of secreting multiple cytokines provide the most effective control of viral infection (64–66). Importantly, both ELISPOT and ICS assays can be used to characterize pools of epitopes in conditions where only small amounts of PBMC are available.

An alternative and complementary approach to ELISPOT and ICS involves the use of tetramer staining reagents (67, 68). This approach requires not only the production of specific reagents for each HLA:epitope combination, but also that T cells specific for each combination are present in sufficient frequency in peripheral blood to allow their detection and characterization. In cases where T-cell frequency is low, this limitation can be overcome by the tetramer enrichment technique (69). Because tetramer characterization is in general more technically demanding, tetramer assays are ideally suited for in-depth characterization of a small but representative set of epitope specificities.

Markers associated with memory or activation/exhaustion states are also of interest. For vaccines to be effective, they must promote development of an effective T-cell memory response, in terms of recall of both effector T-cell responses and anamnestic

antibody responses (70). T cell can be classified into T_N (naïve T cells), T_{CM} (central memory T cells), T_{EM} (effector memory T cells), and T_{EMRA} (effector memory T cells re-expressing CD45RA) subsets (71). Activation and conversely exhaustion of T cells are implicated in the regulation of protective immunity and immunopathology (72). Markers such as CD57 (73, 74), CD40L (75), and PD-1 (programed death-1) (76) allow determination of activation/exhaustion states of memory T-cell subsets. All of these techniques are currently available and a detailed analysis of the responses induced by natural or experimental infection with DENV will greatly contribute to the understanding of T-cell immunity in humans and may further contribute to identify robust correlates of protection in natural immunity and vaccination against DENV.

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Control of acute dengue virus infection by natural killer cells

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Dengue fever is the most important arthropod-borne viral disease worldwide, affecting 50–100 million individuals annually. The clinical picture associated with acute dengue virus (DENV) infections ranges from classical febrile illness to life-threatening disease. The innate immunity is the first line of defense in the control of viral replication. This review will examine the particular role of natural killer (NK) cells in DENV infection. Over recent years, our understanding of the interplay between NK cells and viral pathogenesis has improved significantly. NK cells express an array of inhibitory and activating receptors that enable them to detect infected targets while sparing normal cells, and to recruit adaptive immune cells. To date, the exact mechanism by which NK cells may contribute to the control of DENV infection remains elusive. Importantly, DENV has acquired mechanisms to evade NK cell responses, further underlining the relevance of these cells in pathophysiology. Hence, understanding how NK cells affect the outcome of DENV infection could benefit the management of this acute disease.

Keywords: NK cells, dengue viral infection, cytokines, cytotoxicity, viral escape mechanisms

INTRODUCTION

Dengue virus (DENV) is the most widespread arbovirus worldwide transmitted by mosquitoes of the Aedes genus and is responsible for major outbreaks leading to serious health and economical problems (1). Approximately 500,000 DENV cases progress to be a life-threatening disease each year causing up to 20,000 deaths (2). DENV is a member of the genus Flavivirus and is divided into four different serotypes (DENV1–DENV4). Early 1970, Halstead and Simasthien (3) suggested that primary infection with one serotype of DENV only confers short-term partial cross-protection against other serotypes. Furthermore, secondary heterologous infections contribute to the development of severe forms of dengue fever (DF) [dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS)] (4).

The clinical picture of primary DENV infection includes fever, headache, myalgia, arthralgia, and petechial rash. Patients rapidly develop high viremia for up to 6 days after the onset of fever. The rapid initiation of the hosts' innate defense mechanisms might be a limiting factor in the development of DENV infection. In rare cases, patients may develop complications including plasma leakage and coagulation disorders, which may lead to a fatal outcome (5, 6). One of the most important questions in regards to dengue pathogenesis is the identity of the cells that play a crucial antiviral role during the innate immune response to DENV at the earliest stages of infection (7). Natural killer (NK) cells are a major component of the innate arm of the immune system. Although NK cells hold an early and central role early after number of viral infections, not only for viral containment but also for timely and efficient induction of adaptive responses, their role in the control of DENV infection is still poorly documented. The observation in the late 1970s that viral infections induce NK cell-mediated killing stimulated a frenzy of research focused on evaluating the role of these cells in defense against several viral infections and cancer. The clearest demonstration of this role derives from the growing number of cases of NK cell deficiency. Since the initial known case of a girl with multiple severe or disseminated herpesvirus infections, about 40 other unrelated cases have been described and were mainly associated with severe herpesvirus infections, and EBV- or HPV-related cancers (8). Major progress has been achieved since, in several important fields related to NK cells. NK cells represented a wonderful biological paradox in that they appeared fully competent to kill target cells and yet were clearly self-tolerant. As such, NK cells were "armed" but not dangerous. Scientists focused their attention on understanding how these potent killers were contained and controlled. We now appreciate that a precise balance of inhibitory and activating signals mainly regulates the functional outcome of these cells (9–11). A flood of information concerning multiple types of negative receptors on NK cells was gathered. Many of these receptors respond to stimulation by major histocompatibility complex (MHC) class-I molecules expressed on the surface of target cells and this is considered to be the predominant mechanism responsible for NK cell tolerance to self. These inhibitory NK receptors include ILT-2 and the CD94/NKG2A complex, which recognize

HLA-G and HLA-E, respectively, whereas the inhibitory killer-cell immunoglobulin-like receptors (KIRs) recognize polymorphic MHC class-I molecules; in particular: KIR2DL1/KIR2DL2 and KIR2DL3 bind group 2 (C2) and group 1 (C1) HLA-C alleles, respectively, while KIR3DL1 recognizes HLA-Bw4 epitopes (12). Numerous studies have shown that these factors likely synergize to generate susceptibility or resistance to pathogens and disease (12, 13). In patients from southern Brazil, the susceptibility to DF is positively associated with the presence of KIR3DS1-Bw4, KIR3DL1-Bw4, KIR2DL1-C2, and KIR2DS1-C2 genes, and negatively associated with KIR2DL3-C1/C1 (14).

To destroy a target, NK cells also present several cell-surface activating receptors such as: NKG2D, DNAX accessory molecule-1 (DNAM-1), an adhesion molecule physically and functionally associated to Lymphocyte function-associated antigen 1 (LFA-1), and the natural cytotoxicity receptors (NCRs: NKp30, NKp44, and NKp46) (15, 16). Simultaneous interactions of certain of these activating receptors on NK cells with their specific ligands on the target cells, lead to the integration of different signals including the modulation of the cell-cycle, and together dictate the quality and intensity of the effector NK cell response (17). The relative contribution of each of the activating receptors to NK cytotoxicity against target cells differs, indicating the existence of an array of specific ligands that will be presented and their role in DENV infection further developed by Beltrán and Lopez-Verges, under the research topic "Protective Immune Response to Dengue Virus Infection and Vaccines: perspectives from the field to the bench."

In this mini-review, we focused on what is currently known about the mounting of the NK cell response with its role in cytotoxicity and immunoregulation during DENV infection as well as the mechanisms acquired by the virus to evade NK cell killing.

ACTIVATION OF NK CELLS AFTER DENV INFECTION

Very early after infection, interferon-alpha (IFN-α) is the mainstay of host defenses. This type I, IFN, is a crucial mediator of the antiviral response directly inhibiting viral replication and modulating downstream immune responses to counteract viral spread (18). Elevated IFN-α plasmatic levels are observed shortly after onset of symptoms in children and adult DENV-infected patients (19, 20). Recently, Gandini et al. (21) have shown that DENV2 efficiently activated IFN-α production by plasmacytoid dendritic cells (pDCs), which produced up to 1000-fold more IFN-α than other cell types in response to virus exposure. The infected pDCs could then decrease DENV infection of monocytes. The importance of the IFN-α response is also illustrated by the increased lethality of IFN-α/β receptor knockout mice when administered DENV2 by intraperitoneal injection (22). Although it was shown that the *in vitro* pretreatment of cultured cells with IFN-α/β dramatically reduces DENV replication, type I IFN has little effect on DENV replication after viral replication has been established (23). Indeed, DENV can reach high titers ($<10^9$ infectious doses per milliliter) in humans despite the induction of high levels of circulating IFN-α (19, 24). Therefore, it seems likely that DENV has evolved mechanisms to counteract the IFN response, a characteristic that is shared by many pathogenic viruses (25).

It has long been established that one of the main mechanisms accounting for the efficacy of the type I IFNs is their ability to activate NK functions (**Figure 1**); thus, they promote the accumulation and/or survival of proliferating NK cells by the STAT1-dependent induction of IL-15 secretion (26). This early activity of NK cells may be important for clearing primary DENV infection. In a sensitive mouse model, acute infection with DENV showed a rapid increase of NK cell levels (27). A significant increase in the frequency of NK cell circulation was also shown in patients who developed an acute DF (28). In addition, patients with a mild DF have elevated NK cell rates when compared to those with severe DF (29). Interestingly however, levels of circulating MIP-1β are higher in mild acute DF and are associated with higher NK cell frequencies (30). To characterize the primary NK response to DENV infection in mice, the phenotype of NK cells in the spleen was assessed by flow cytometric analysis. Three days after infection, the DENV-infected mice had twice as many NK cells than the mock-infected mice, and more than 50% of these NK cells expressed the early activation marker CD69, although only 5–10% of NK cells in the mock-infected animals expressed CD69 (27). Concomitantly, NK cells from DENV-infected patients display simultaneously high levels of CD69, HLA-DR, and CD38 (28, 29). For example, a significant increase in the percentage of $CD69^+$-expressing NK cells was observed in DENV-infected patients at the early and acute phase of infection (days 1–5 with 29 vs. 13%), maintained at days 6–10 (24 vs. 18%), but decreased after 11 days (13 vs. 5%) (28). Altogether, these observations support the concept that DENV infection induces the selection and proliferation of a subset of activated NK cells further reinforcing their potentially important role during the early stages of the disease.

NK CELL FUNCTION IN THE PROTECTION AGAINST DENV

CYTOKINES PRODUCED BY ACTIVATED NK CELLS

Upon activation, NK cells may produce cytokines that favor the complete elimination of the disease and the infectious agent during the adaptive response, such as IFN-γ, TNF-α, G-CSF, and GM-CSF, as well as chemokines, such as MIP-1α, MIP-1β, and Rantes (31) (**Figure 1**). Numerous investigations have shown that DENV-infected patients presented significantly elevated levels of IFN-γ, G-CSF, and GM-CSF (20, 29, 32), whereas other reports suggest that TNF-α elevation could be associated with disease severity (33, 34). However, the results were obtained by measuring cytokine levels in sera collected from DENV-infected patients, and do not reveal which specific cells are activated and involved in their production. To date, the role played by NK cells in the production of these soluble factors is unknown. Deeper investigations will be necessary to precisely determine the implication of the immune-regulation by NK cells during the acute DENV infection.

NK CELL AND THEIR RECEPTORS DURING DENV INFECTION

One of the most prominent functions of NK cells is the capacity to lyse virus-infected cells. When the balance of inhibitory/activating signals is in favor of activation, the engagement of activating receptors on the surface of NK cells leads to directed exocytosis of granules containing perforin and granzymes, which in turn elicit the disruption of the target cell membrane and/or the activation of apoptosis pathways within the infected cell (11). It has been shown that activated NK cells are cytotoxic against DENV-infected cells (35). Furthermore, a marker of cytotoxicity, the granule cytotoxic

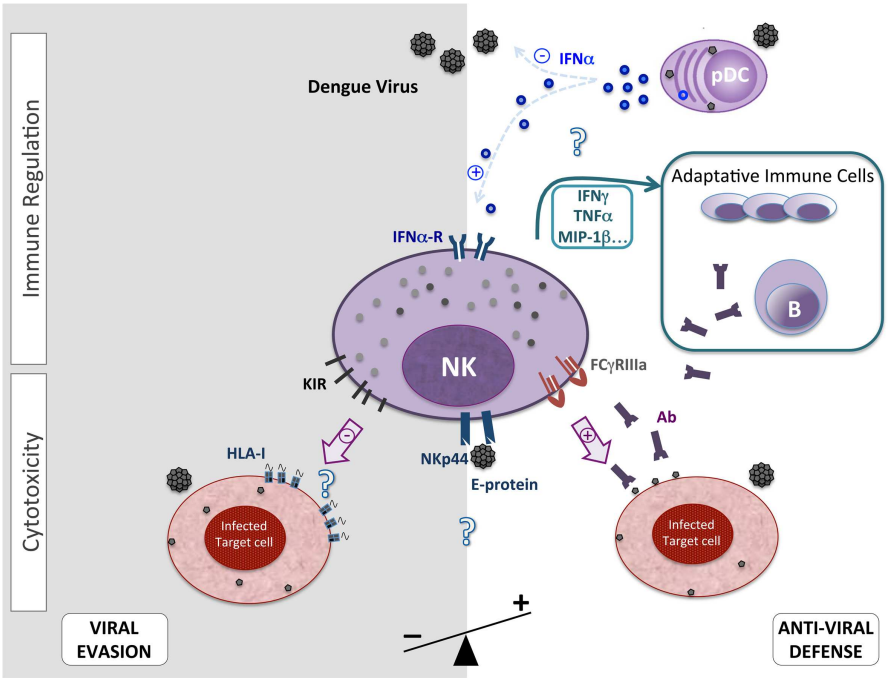

FIGURE 1 | Overview of the suggested NK cell features after acute and primary DENV infection.

T cell intracellular antigen TIA-1, as well as two adhesion molecules, CD44 and CD11a, both involved in NK cell migration to various tissues and NK cytotoxicity, have been found to be significantly elevated on NK cells collected from acutely infected patients (28).

Hershkovitz et al. (36) have demonstrated the existence of a direct protein–protein interaction between recombinant DENV soluble envelope E protein and NKp44 (but not NKp30 or NKp46) that could be involved in the triggering of cytolysis (**Figure 1**). Using West Nile virus (WNV) like particles (VLPs) and WNV-infected cells, they have shown that E–NKp44 interaction triggers the secretion of cytotoxic granules contained in NK cells, the lysis of target cells, and the increased production of IFN-γ suggesting that flavivirus E proteins activate NK cytotoxic activity through NKp44 engagement.

Overall our understanding of the mechanisms involved in the cytolysis of target DENV-infected cells by NK cells is in its infancy. The receptors and signaling pathways essential to this major function are yet to be clearly identified. Recent investigations focusing on the various target ligands involved in the cytotoxic response by Beltrán and Lopez-Verges, under the Research topic "Protective Immune Response to Dengue Virus Infection and Vaccines: perspectives from the field to the bench" may bring some insight as to these questions.

ACTIVATION OF NK CELLS BY ADCC RESPONSES DURING EARLY DENV INFECTION

Antibody-dependent cell-mediated cytotoxicity (ADCC) is another known mechanism by which NK cells recognize and lyse antibody-coated target cells, through the engagement of antibody binding to the Fc gamma receptor IIIA (CD16) (37). In an *in vitro* model, Kurane et al. (35) reported that human blood NK cells are cytotoxic against DENV-infected cells via direct cytolysis but also via ADCC (**Figure 1**). Indeed, PBMC collected from a DENV-infected patient successfully lysed DENV-infected cells that did not express MHC class-I molecules, and lysis of infected cells was significantly increased upon addition of anti-DENV1 and DENV2 monoclonal antibodies. The addition of sera from an individual without DENV antibodies did not lead to an increase in lysis of infected cells. More recently, García et al. (38) tested acute and convalescent patients' sera for ADCC activity, differentiating mild and severe forms of DF. ADCC activity was observed with acute sera only in cases of DHF/DSS but not DF. However, using convalescent sera collected 1 year later, all samples induced ADCC activity. This suggests that the development of ADCC activity during the acute phase of the infection could be associated to the pathological manifestations of the severe syndrome and that the systematic development of ADCC activity after a primary infection, in convalescent patients could be associated with the development of severe forms during a subsequent heterologous DENV infection. However, other studies suggest a protective role for ADCC against DENV secondary infections. Indeed, higher ADCC activities have been associated with higher plasma neutralizing antibody levels and lower viral loads during secondary infection but solely if the patient was secondarily infected by DENV3 and not DENV2 (39).

Altogether, this data support a strong implication of ADCC after DENV infection, however, careful investigations are needed to determine its exact contribution both in the viral clearance during the initial acute phase of this infection and DENV pathogenesis in secondary infections.

DENV EVASION OF NK CELLS

Co-existence of viruses and their infected hosts imposes an evolutionary pressure on both the virus and the host's immune system. The host has developed an immune system able to attack viruses and virally infected cells, and viruses have developed an array of immune evasion mechanisms to escape being killed by the host's immune system (40, 41). A wide variety of viruses have developed mechanisms to evade the NK cell response, and among these the flaviviruses are known to have developed particularly evolved mechanisms to escape NK cell-mediated lysis. Many viruses evade T-cells recognition by down-regulating MHC class-I restricted antigen presentation, whereas flaviviruses induce their cell-surface expression. This leaves infected cells less susceptible to NK lysing, MHC class-I molecules being able to effectively engage NK cell inhibitory receptors (11, 42, 43). Regarding DENV infection, a number of studies have demonstrated that DENV replicon expression is sufficient to enhance membrane expression of MHC class-I inducing a reduced susceptibility to NK lysis (42, 44). The way by which DENV induces MHC class-I up-regulation on the surface of infected cells has been associated with an activation of NF-κB, independently of IFN and without an increased synthesis of MHC class-I molecules (45). The exact mechanism(s) and especially the viral components involved are still obscure yet several hypotheses were proposed. The up-regulation of MHC class-I expression could be the result of: (i) an increase in the supply of peptides to the endoplasmic reticulum mediated by the transporter associated with antigen processing (TAP) (46) (ii) the expression of DENV non-structural protein(s) and/or viral RNA replication (44), and (iii) the accumulation of uncleaved C-prM protein during viral assembly (47).

The major biological consequence of DENV-induced MHC class-I increase is a profound reduction of NK cell lysis as shown in *in vitro* cytotoxicity assays (35, 44). Moreover, an aggregation of MHC class-I molecules at the surface of infected cells seems to lead to increased affinity with NK cell inhibitory receptors (44). Given that the kinetics of DENV viremia coincide with the peak of NK cell response, the modulation of MHC class-I expression may have physiological relevance in counteracting host NK cell defense. It is likely that there is a complex balance between DENV-induced activation of NK cells and subversion of NK killing by DENV-induced MHC class-I expression on infected cells. An additional component to take into account is the genetic background of the host since KIRs/HLA molecules combinations play a crucial role in the strength of NK cell inhibitory function (13), and this could clearly play important role on DENV infection susceptibility, protection, and severity.

These viral escape mechanisms certainly reflect evolutionary pressure exerted by the host immune response on the pathogen but also highlight the importance of NK cells in the defense against DENV infection.

CONCLUSION

Overall, several lines of evidence converge to suggest that NK cells are activated early during an acute DENV infection, and produce major cytokines, such as IFN-γ, which participate in the control of the viral replication while promoting the development of an efficient adaptive immune response. In the future, it will be interesting to determine if and how DENV leaves an imprint on the NK receptor repertoire possibly favoring cells with a strong cytolytic potential, as previously shown, by us and other groups, with several other viruses, including chikungunya virus, which is yet an other arbovirus (48). Numerous studies have also shown that DENV increases MHC class-I and adhesion molecule expression, allowing the virus to escape NK cell lysis. Whilst underlining the importance of NK cells in DENV infection, these observations must be interpreted with great caution. Due to the lack of extensive phenotypic and functional studies in DENV-infected patients, we cannot conclude in favor of a beneficial or a deleterious role of the NK cells in the control and/or the evolution of the disease. Future studies based on the depletion of NK cells in a relevant animal model for DENV infection, will be able to unravel this fascinating topic.

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NK cells during dengue disease and their recognition of dengue virus-infected cells

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The innate immune response, in addition to the B- and T-cell response, plays a role in protection against dengue virus (DENV) infection and the degree of disease severity. Early activation of natural killer (NK) cells and type-I interferon-dependent immunity may be important in limiting viral replication during the early stages of DENV infection and thus reducing subsequent pathogenesis. NK cells may also produce cytokines that reduce inflammation and tissue injury. On the other hand, NK cells are also capable of inducing liver injury at early-time points of DENV infection. *In vitro*, NK cells can kill antibody-coated DENV-infected cells through antibody-dependent cell-mediated cytotoxicity. In addition, NK cells may directly recognize DENV-infected cells through their activating receptors, although the increase in HLA class I expression may allow infected cells to escape the NK response. Recently, genome-wide association studies have shown an association between *MICB* and *MICA*, which encode ligands of the activating NK receptor NKG2D, and dengue disease outcome. This review focuses on recognition of DENV-infected cells by NK cells and on the regulation of expression of NK cell ligands by DENV.

Keywords: dengue, NK cell, NK receptor, NK ligand, innate immune response

INTRODUCTION

Dengue is a major public health problem in tropical and subtropical regions world wide and is caused by four serotypes of dengue virus (DENV-1, -2, -3, -4), a flavivirus transmitted to humans by *Aedes* mosquitoes (1). DENV infection can be asymptomatic or induce mild to severe disease, traditionally referred to as dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), which can lead to death (2). While there have been important advances in elucidating dengue pathogenesis, it is difficult to foretell if an acutely infected individual will develop the disease and to predict its severity; however, disease outcome appears to depend on the virus as well as on host genetics and prior immunity (3–5). For this reason, it is crucial to understand the immune response during DENV infection. Many studies have focused on the adaptive response, as antibodies and T cells play a crucial role in protection against infection, as well as in the pathogenesis of dengue disease (4–6). The innate immune response plays an intrinsic role at the level of the infected cell (7), but also recruits and activates innate immune cells that can eliminate the virus at early stages and induce the development of the adaptive response (8, 9). Indeed, the extent of DENV replication during the early period of infection correlates with dengue disease severity (10–14). Interstitial dendritic cells (DCs) are believed to constitute the first line of host defense against invading DENV at the anatomical sites where it replicates after the initial bite by infected mosquitoes (8). Type-I interferon-dependent immunity is known to play a critical role, and early activation of natural killer (NK) cells may also be important in limiting viral replication at the early stages of DENV infection (6, 8).

NK CELLS IN VIRAL INFECTIONS

Natural killer cells are innate lymphocytes specialized in defense against viral and intracellular bacterial infections and tumors (15). NK cells share some characteristics with the adaptive immune system and may possess specific memory features against some viruses and antigens (16). They can be rapidly recruited into infected organs and tissues by chemoattractant factors produced by virus-infected cells and activated resident macrophages and DCs, which are a major source of the interferon IFNα/β that induces NK cell proliferation and activation (17, 18). Reciprocally, NK cells can shape DCs activation and subsequently the adaptive response (17). Once activated, NK cells fight infection by producing chemokines and anti-viral cytokines, mainly IFNγ and MIP1-β, and by recognizing and eliminating infected cells by antibody-dependent cell-mediated cytotoxicity (ADCC) or by direct recognition through their activating receptors (15). NK cells have activating and inhibitory receptors that allow them to recognize stressed cells, tumors, and pathogen-infected cells and to differentiate them from healthy cells (15, 19). Most of the inhibitory receptors recognize classic and non-classic major histocompatibility complex class I (MHC I) molecules, and many viruses decrease the expression of MHC I molecules in infected cells to escape the $CD8^+$ T-cell response, thereby becoming more vulnerable to NK cell recognition (19–21).

Virus-infected cells often induce or increase the expression of ligands at their surface, allowing for recognition by NK cell activating receptors, including NKG2D, DNAM-1, CD94-NKG2C; the NCR receptors NKp46, NKp30, NKp44; and others (19, 20, 22). The ligands include host stress-induced molecules and viral

Table 1 | Summary of the published findings related to ligands for receptors on NK cells and dengue virus infection.

Ligand	Study type	Observation	Receptor on NK cells	Probable effect	Reference
ICAM-1	Acute patients' lymphocytes	Adhesion molecules (ICAM-1 and LFA-1) are increased in NK cells in the acute phase of dengue disease	LFA-1	adhesion	Azeredo et al. (31)
DENV-specific antibodies	*In vitro* with activated peripheral blood lymphocytes	NK cells are the principal cells active in the ADCC against DENV-infected cells	CD16	Activation, ADCC	Kurane et al. (42, 43) and Laoprasopwattana et al. (44)
DENV ligands (probably: protein E)	*In vitro* activation of NK cell toward E protein and VLP of flaviviruses	Protein–protein interaction between rNKp44 and cells expressing DENV-E proteins. Interaction of NKp44 with E from another flavivirus (WNV) induces killing and IFNγ production	NKp44	Activation?	Hershkovitz et al. (55)
MICA	Allele association	MICA alleles associated with dengue symptomatic infection	NKG2D	Activation?	García et al. (59)
MICB	Allele association, GWAS	MICB alleles associated with symptomatic infection and dengue shock syndrome	NKG2D	Activation?	García et al. (59), Khor et al. (60), Whitehorn et al. (61)

proteins (20). To date, the ligands for many of the activating receptors (for example the NCRs) are still unknown, and their expression has been detected indirectly by cell staining with recombinant receptors or by blocking of killing with receptor-specific antibodies. A better characterization of NK ligands is needed. NKG2D is the most well-characterized NK activating receptor, and ligand binding leads to target killing and cytokine production (22). NKG2D ligand expression is increased by "stress" conditions, including viral infections (22, 23). In humans, eight ligands have been described for NKG2D, including MICA, MICB, and ULBP1-6. In addition to expression of NKG2D ligands on the surface of infected cells, soluble isoforms can be released into the serum, although the physiological relevance of the soluble ligands is controversial (22, 24). These soluble forms have been described in cancer patients, as well as in HIV-1-infected patients without therapy (25, 26) suggesting that soluble NKG2D ligands might be released in other viral infections.

ROLE OF NK CELLS IN DENGUE DISEASE

Many studies suggest that NK cells play a role in the response against DENV infection, principally in the early infection stages by limiting DENV replication. A higher absolute number of NK cells was observed in patients with mild dengue fever (DF) compared with children who developed DHF (27–29). However, the percentage of NK cells and $CD8^+$ T cells expressing CD69, a marker of activation, was higher early during infection of children who developed DHF (27, 30). Homchampa et al. found evidence of NK cell cytotoxicity against non-infected K562 target cells from children with acute dengue, and the cytolytic activity was increased on a per-cell basis in the early disease stages of dengue compared with healthy controls, and was even higher in the most severe form of the disease. It was suggested that this NK cell activity was associated with higher viremia in the more severe cases. Studies in adults showed that patients with mild disease had higher numbers of NK cells, with the majority of cells having increased expression of activation markers (CD69, CD38, and HLA-DR), adhesion molecules (CD11a and CD44) (**Table 1**), and markers of intracellular cytotoxic granules (TIA-1), in contrast to severe dengue where reduced NK cell numbers were observed (31). The authors suggest that higher NK cell percentages and activity might indicate a good prognosis of disease. In a genome-wide association study (GWAS), it was shown that the transcriptome of blood cells from children with DSS was characterized by decreased abundance of transcripts related to T and NK responses, probably not due to a difference in lymphocyte counts but to an impaired response (32). The differences observed in NK cell numbers and percentages in these studies may be explained by differences in age (children versus adults), ethnicity, time of infection, and the experimental methods to define NK cells and their activation. Nonetheless, all these observations point to the importance of NK cells and their activation during early DENV infection. The activation and phenotype of NK cells during dengue acute infection is further developed by Petitdemange et al., under the research topic "protective immune response to dengue virus infection and vaccines: perspectives from the field to the bench."

The protective role of NK cells in the response against DENV is supported in mice models of the disease. In immunocompetent A/J mice, the early activation of NK and B cells was associated with the control of the viral load and the prevention of disease (33, 34). In C57BL/6 mice, the recruitment of NK and NKT cells by mast cells to the site of infection was also crucial for viral clearance (35), underlining the importance of these cells during the host early response against DENV. The early recruitment of NK cells to the liver was induced in part by CXCL10 (IP-10), and in the liver NK cells produce effector molecules (perforin, granzyme A, and granzyme B) needed for viral clearance (36). On the other hand,

NK cells appear to play not only a protective role in dengue disease, as it was shown that after intrahepatic infiltration, NK cells were responsible for cell death in the liver at the early phase of infection, whereas $CD8^+$ T cells were responsible for damage later (37). The mechanism of this cell death has not been elucidated, but the authors suggested that NK cells were killing DENV-infected cells. It is possible that under some conditions, the elimination of DENV-infected cells by NK cells can be exacerbated in a way that the immune effector cells become responsible for organ injury. Similarly, NKT cells in some conditions can be detrimental during dengue pathogenesis, in part by inducing NK cells and neutrophils activation (38).

Natural killer cells may play an important role in early DENV infection *in vivo* also by producing together with γδ T cells, IL-22 and IL-17A, which may influence dengue disease outcome (39). NK, NKT, and T cells that can respond in a non-TcR-dependent fashion are a major source of IFNγ in immune responses induced by inactivated DENV (40); however, in this context, the frequency of IFNγ-producing cells observed was very low. Using C57BL/6 mice, a study confirmed the role of NK cells in the early IFNγ production induced by IL-12 and IL-18 in response to DENV, key for the control of viral load and DENV-2-associated disease severity and lethality (41).

Natural killer cells can mediate ADCC against DENV-infected cells (42, 43) and this mechanism may be important during secondary infections when antibodies to DENV are present. Indeed, ADCC activity in plasma obtained before secondary DENV-2 or DENV-3 infection correlated with serotype-specific neutralizing antibody titers, anti-DENV IgG1 levels, and a multitypic $PRNT_{50}$ pattern (44). Interestingly, a higher level of ADCC activity measured before secondary DENV-3 infection was associated with lower subsequent viremia, which suggests a protective role for antibodies and NK cells; however, this association was not observed for secondary DENV-2 infection. In another study, ADCC was correlated with DENV surface antigen expression, suggesting recognition by anti-DENV antibodies (42), whose Fc region is then recognized by the activating low affinity CD16 (FcγRIII) receptor on NK cells (45).

Human NK cells ($CD3^-CD16^+CD11b^+$ cells) can lyse DENV-infected cells to a greater level than uninfected cells even in the absence of antibodies, suggesting a mechanism of direct recognition as well (42, 43). These studies were performed using DENV-infected Raji cells as targets. The NK receptors and their ligands implicated in the direct recognition of DENV-infected cells have not been fully elucidated, indicating the need for future studies.

EXPRESSION OF NK CELL LIGANDS DURING DENGUE DISEASE

Viruses try to escape the immune response of the host. As NK cells are crucial players in the anti-viral response, many viruses induce the up-regulation of MHC I, which serve as ligands for NK inhibitory receptors, in order to dampen the NK cell response even if enhancing expression of MHC I might increase their recognition by $CD8^+$ T cells (20). DENV and other flaviviruses induce the up-regulation of MHC I (46–51). Expression of flavivirus pr-M protein in hamster cells induced increased surface expression of MHC I, although the authors suggested this could be an incidental consequence of viral assembly rather than a specific mechanism of immune evasion (52). It has also been shown that human cell lines expressing the non-structural (NS) proteins of DENV up-regulate MHC I (HLA-A, -B, -C) expression at their surface by TAP-dependent and TAP-independent pathways (46). This resulted in a lower sensibility to lysis by NK cells, probably due to recognition by the corresponding inhibitory receptors (e.g., KIR2DL1 for HLA-C) and the inhibitory receptor LIR-1 that recognizes all MHC I proteins (**Table 1**). Further studies are needed to determine if MHC I up-regulation by DENV is the response to a particular viral protein, viral replication itself, or type-I IFNs induced by viral infection, and whether it plays a role in immune evasion of NK cells *in vivo*. Neurotropic flaviviruses, such as West Nile virus (WNV), can transiently activate and then suppress NK cell activity (53). Future studies are needed to determine if this happens during DENV infection by analyzing NK cell activity at different time points post-infection.

Even if DENV NS proteins induce MHC I up-regulation, NK cells can kill DENV-infected cells (28, 42, 43), suggesting that the signals for NK cell activation overcome the inhibitory signals. As described above, NK cells can kill DENV-infected cells by ADCC mediated by CD16 (42, 44) (**Table 1**), which is expressed on resting human NK cells and can induce a strong activating signal leading to cytolysis (20, 54). Interestingly, NK cells can also kill DENV-infected cells in the absence of antibodies (42), implicating a direct recognition by NK cell activating receptors. It has been reported that the activating receptor NKp44 can interact with the envelope protein (E) of DENV (55) (**Table 1**). NKp44 has also been implicated in killing WNV-infected cells after blocking the inhibitory receptor LIR-1 on NK cells, and this also induced IFNγ production. Further studies are necessary to determine if recognition of DENV-E by NKp44 on NK cells can induce a similar response against DENV-infected cells. NKp44 is expressed on activated, but not resting, NK cells (56) and can trigger NK cell killing of both tumor and virus-infected cells (57, 58).

Recently, a sequencing-based typing method and genotyping of asymptomatic DF and DHF patients in Cuba uncovered an association of certain alleles of the *MICA* and *MICB* genes (*MICA*008* and *MICB*008*) with symptomatic DENV infection (59) (**Table 1**). The importance of MICB in dengue susceptibility was also indicated by GWAS with a large number of pediatric cases in Vietnam, where certain *MICB* and *PLCE1* alleles showed a significant association with DSS (60). These results were confirmed by a study showing that *MICB* rs3132468 and *PLCE1* risk genotypes were also associated with less severe clinical phenotypes of dengue in adults as well as with DENV infection in infants (61). This strongly suggests a role for this *MICB* variant in susceptibility to overall clinically apparent dengue disease. It still has not been determined whether the association between these NKG2D ligands and dengue clinical responses is directly due to the function of MICA/B molecules in dengue pathogenesis. Nonetheless, the importance of NKG2D ligands in the NK cell response against other viral infections (22) supports this hypothesis. Given the role of MICB in activation of NK, NKT, and $CD8^+$ T cells through the NKG2D receptor, these findings support a central role for these cell types in shaping the outcome of DENV infection. It

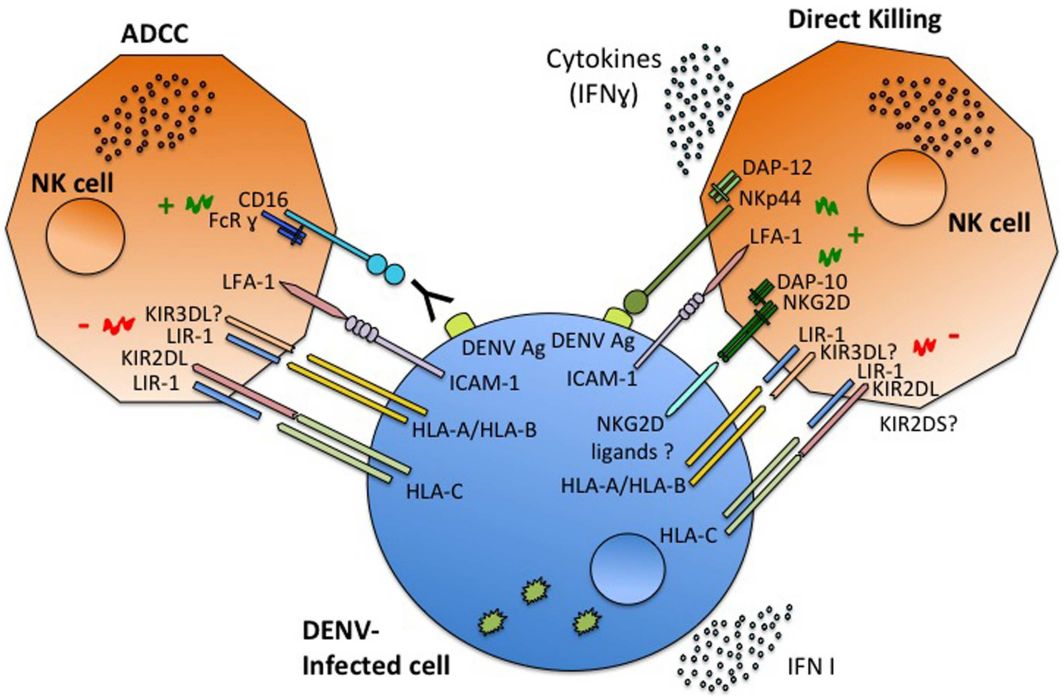

FIGURE 1 | Schematic model of NK cells interaction with DENV-infected cells. NK cells recognize infected cells through the interaction of their receptors with ligands on the infected cells, the outcome depends on the balance between activating or inhibitory signals. Once activated NK cells are capable of ADCC or direct cytotoxicity, and of producing cytokines, mainly IFNγ.

is plausible that the *MICB* risk-associated phenotype is associated with an impaired NK cell response, potentially resulting in a higher *in vivo* virus titer and an increased risk of developing both symptomatic and severe dengue. Furthermore, inefficient induction of cytokines secreted by NK cells might result in dysregulated T-cell responses that may also shape the clinical phenotype (62). NKG2D ligand (MICA/B) expression on DENV-infected cells may allow direct recognition by NK cells, which might be important for the early innate immune response against DENV infection, leading to either more effective control of viral infection or alternatively contributing to the disease pathology. The expression pattern of these NKG2D ligands in DENV-infected cells *in vitro* and *in vivo* needs to be determined. It is also necessary to determine if in acute viral infections such as DENV, the production of soluble NKG2D ligands can also be observed and if this impacts dengue clinical manifestations. Because ligands of other NK activating receptors, such as DNAM-1, can be induced by "stress" (23), further studies are needed to characterize all the NK receptor ligands induced during DENV infection, and the results may depend on the cell-type analyzed, as well as the DENV serotype. A complete characterization of DENV-infected cell recognition by NK cells (**Figure 1**) is crucial to better understanding the role of these cells in dengue disease.

CONCLUSION

The innate immune response, and particularly type-I IFN and NK cells, plays a key role during the early infection events due to its ability to rapidly limit viral dissemination and to affect the antigen-specific, adaptive immune responses to effectively clear pathogens (8, 9). More studies in animal models and human populations will enable deciphering NK cell responses during DENV infection *in vivo*. Furthermore, *in vitro* experiments will also be needed to determine which NK receptors and ligands are implicated in DENV-infected cell recognition and NK cell-mediated killing and cytokine production. To date, only the up-regulation of MHC I and the induction of a putative NKp44 ligand (DENV-E protein) have been reported in DENV-infected cells. Are there other ligands that play a role in NK cell recognition induced during DENV infection? Genetic studies suggest an important role for the NKG2D ligands MICA and MICB; however, as yet no functional experiments have validated this hypothesis. There is still much to do to determine if ligands of other NK receptors, for example HLA-E for CD94/NKG2A-C and the ligands for the activating receptors 2B4 and DNAM-1, are induced or repressed during DENV infection, and whether this has an implication in the immune response to DENV and its clinical outcome. Finally, the characterization of NK receptor ligands and the NK cell phenotype in patients' blood cells will provide insights into the NK cell subsets activated during dengue disease. It will also establish possible associations between NK cell activity or NK cell ligand expression and protection from disease and/or increased dengue severity. Whether DENV interferes with innate anti-viral immunity mediated by NK cells at early times of infection and whether DENV virulence might be associated with its ability to counter the host cell defenses are critical issues that remain to be elucidated.

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Dendritic cells in dengue virus infection: targets of virus replication and mediators of immunity

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Dendritic cells (DCs) are sentinels of the immune system and detect pathogens at sites of entry, such as the skin. In addition to the ability of DCs to control infections directly via their innate immune functions, DCs help to prime adaptive B- and T-cell responses by processing and presenting antigen in lymphoid tissues. Infected *Aedes aegypti* or *Aedes albopictus* mosquitoes transmit the four dengue virus (DENV) serotypes to humans while probing for small blood vessels in the skin. DENV causes the most prevalent arthropod-borne viral disease in humans, yet no vaccine or specific therapeutic is currently licensed. Although primary DENV infection confers life-long protective immunity against re-infection with the same DENV serotype, secondary infection with a different DENV serotype can lead to increased disease severity via cross-reactive T-cells or enhancing antibodies. This review summarizes recent findings in humans and animal models about DENV infection of DCs, monocytes, and macrophages. We discuss the dual role of DCs as both targets of DENV replication and mediators of innate and adaptive immunity, and summarize immune evasion strategies whereby DENV impairs the function of infected DCs. We suggest that DCs play a key role in priming DENV-specific neutralizing or potentially harmful memory B- and T-cell responses, and that future DC-directed therapies may help induce protective memory responses and reduce dengue pathogenesis.

Keywords: dengue virus, dendritic cells, monocytes, macrophages, innate immunity, antibody-dependent enhancement, immune evasion

INTRODUCTION

DENDRITIC CELLS, MONOCYTES, AND MACROPHAGES: LOCATION AND FUNCTION

Dendritic cells (DCs) reside and migrate into barrier tissues such as the skin and mucosal epithelium that are the sites of pathogen invasion. In the steady state, DCs display high levels of phagocytic activity, take up antigen, and probe for pathogens via pattern-recognition receptors. DCs express Toll-like receptors (TLRs) and C-type lectins as transmembrane proteins as well as intracellular sensors, such as retinoic acid-inducible gene I (RIG-I), melanoma differentiation factor 5 (MDA-5), and cyclic GMP-AMP synthase (cGAS) that recognize conserved microbial patterns (1, 2). Upon pathogen recognition, DCs become activated, produce inflammatory cytokines and chemokines, migrate to lymph nodes (LNs), and present antigen to prime naïve T-cells (3).

Subsets of DCs, monocytes, and macrophages (MΦs) reside in different tissues and fulfill distinct functions. Classical DCs (cDCs) display the characteristic DC dendrites, are present in LNs, spleen, and bone marrow as well as skin, lung, liver, and intestine, and have the greatest ability to stimulate naïve T-cells (4). All cDCs can present endogenous antigen from the cytosol via MHC I to $CD8^+$ T-cells (5). In addition, most subsets of cDCs present exogenous antigen via MHC II to $CD4^+$ T-cells, whereas only specialized subsets can cross-present exogenous antigen via MHC I to $CD8^+$ T-cells (6, 7). In addition, plasmacytoid DCs (pDCs) are another DC subset that reside in the spleen, bone marrow, and liver and circulate in the blood. During viral infections, pDCs migrate to infected tissues and secrete up to 1,000-fold higher amounts of interferon (IFN)-α/β than other cell types (8), although their capacity for antigen presentation is still debated (9). Nevertheless, the role of DCs in priming protective immune responses against many human pathogens and their potential contribution to pathogenesis and development of disease need further investigation.

Monocytes circulate in steady-state blood, patrol lymphoid, and non-lymphoid organs, and are recruited to inflamed tissues, where they phagocytize pathogens as well as infected or damaged cells (10). During inflammation, monocytes can differentiate to monocyte-derived DCs (moDCs) (11, 12). *In vitro*-generated human moDCs are used widely to study DC biology (13). Monocytes are isolated from human peripheral blood, differentiated in the presence of GM-CSF and IL-4 first to immature moDCs and after further stimulation with inflammatory cytokines or pathogen-associated microbial patterns (PAMPs) to mature moDCs (13, 14). The ability of moDCs to prime naïve T-cell responses remains controversial, as this function initially was attributed solely to cDCs (15). Nevertheless, recent studies demonstrated that moDCs can migrate to LNs and prime naïve

T-cells during *Leishmania major*, influenza virus, and bacterial infections (12, 16, 17). In contrast to DCs, MΦs have limited ability to migrate and prime naïve T-cells. MΦs reside within tissues, where they phagocytize, secrete cytokines, present antigen to effector and memory T-cells, and contribute to the healing of injured tissue (18).

DENGUE EPIDEMIOLOGY AND PATHOGENESIS

Female *Aedes aegypti* and *Aedes albopictus* mosquitoes transmit the four dengue virus serotypes (DENV1–4) while feeding on blood vessels in the skin (19). The positive-sense RNA genome of the flavivirus DENV encodes three structural (C, prM/M, E) and seven non-structural (NS) proteins (20). DENV causes the most prevalent arthropod-borne viral disease of humans, with an estimated 390 million infections and 96 million apparent cases per year (21).

The acute febrile illness dengue fever (DF) can progress to a potentially life-threatening vascular leakage syndrome, dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), the latter characterized by hypotension and circulatory failure (22). At present, no vaccine or therapeutic against dengue is approved for use in humans. A major challenge in the development of vaccines and therapies is that although infection with one DENV serotype leads to long-lasting immunity against the same serotype, subsequent infection with a different (heterotypic) serotype is the major risk factor for severe disease (19). To date, the mechanisms by which the host immune response to DENV provides either protection or enhancement in secondary infection remain poorly understood. Antibodies can neutralize infection or conversely trigger "antibody-dependent enhancement" (ADE) (23, 24), whereby cross-reactive anti-DENV antibodies facilitate entry of DENV into Fcγ receptor (FcγR)-bearing cells and thus increase viral load and ultimately disease severity. Some DHF/DSS cases occur during primary (1°) infections, especially in infants 6–9 months of age (25). In this case, it is thought that maternal DENV-specific antibodies transferred via the placenta wane to levels that can enhance a newly acquired DENV infection (26). Thus, the quantity and quality of the antibody response influences the severity of a secondary DENV infection. Similarly, T-cells can provide protection (27–29), but cross-reactive T-cells have been implicated in disease pathogenesis (30–32). Nevertheless, most secondary DENV infections are asymptomatic or mild, suggesting that the immune system can mount protective responses against dengue.

Aedes mosquitoes that take a blood meal from a human with acute dengue viremia become infected and, after DENV spreads to the salivary glands, transmit the virus when feeding on a new individual. Mosquito saliva contains components that counteract the host hemostatic response and modulate immunity (33, 34). The addition of saliva from *Ae. aegypti* mosquitoes was found to decrease DENV infection of moDCs *in vitro* (35). In contrast, mosquito saliva or transmission via infected mosquitoes prolonged DENV serum viremia and fever in "humanized" mice as compared to inoculation with DENV alone (36). Furthermore, saliva that was inoculated by non-infected mosquitoes prior to needle inoculation of DENV blocked the upregulation of genes involved in innate pathogen recognition and increased serum viremia in mice deficient in IRF3 and IRF7 (37). Although certain *in vivo* studies suggest that mosquito saliva can facilitate DENV infection by generating an environment that favors early virus replication, the impact of saliva on skin DCs requires further study.

TARGETS OF DENV REPLICATION

DENV INFECTION IN THE ABSENCE OF ENHANCING ANTIBODIES

Identifying the targets of DENV infection is crucial for understanding virus spread and disease pathogenesis. Human autopsies revealed staining for DENV structural proteins and negative-sense viral RNA, indicative of virus replication, in MΦs in LNs, spleen, lung, and liver and monocytes in clotted blood from patients with lethal dengue disease (38, 39). Staining of the non-structural protein NS3 confirmed DENV replication in phagocytes (including monocytes, MΦs, and DCs) in LNs and spleen, as well as in MΦs in the lung in other autopsy studies (40, 41). In earlier stages of the disease, most DENV-infected cells in the peripheral blood of acute dengue patients were identified as $CD14^+$ $CD11c^+$ activated monocytes, with higher proportions of monocytes and DENV-infected total cells in the blood in DHF compared to DF patients (42).

Although in humans, DENV efficiently suppresses the IFN response, replicates, and causes disease, DENV fails to antagonize mouse IFN responses, and thus wild-type (WT) mice generally do not sustain DENV replication or develop disease (43). In comparison, mice deficient in IFN-α/β receptor ($Ifnar^{-/-}$) and also -γ receptor (AG129) are susceptible to DENV infection and display a tropism similar to humans (40, 44–46). DENV replicated in murine MΦs that were isolated from the peritoneum (47), as well as in MΦs in LNs and spleens of AG129 mice (48, 49). Treatment with clodronate liposomes that deplete monocytes and MΦs decreased viral load in AG129 mice on day 2 but increased viral load on day 4 post-inoculation with DENV2 (48). Monocytes and MΦs thus play an important role as targets for early DENV replication as well as in subsequent control of DENV infection.

Human moDCs generated *in vitro* support DENV infection (50), with immature moDCs being more susceptible to DENV infection than mature moDCs, monocytes (51), or MΦs (52). Analogously, $CD11c^{high}$ cells in the spleen of AG129 mice (47) that likely comprised both moDCs and cDCs supported DENV replication *in vivo*. Recent studies in $Ifnar^{-/-}$ mice have shown that early after infection, monocytes are recruited to the dermis and differentiate to moDCs, where they become primary targets for DENV replication (53). Although DENV can infect monocytes and MΦs directly, these studies emphasize the greater permissiveness of DCs to DENV infection in the absence of enhancing antibodies, such as during 1° infection conditions.

Surface expression of viral attachment factors determines the susceptibility to DENV infection. DC-SIGN (dendritic-cell-specific ICAM3-grabbing non-integrin, CD209) is a C-type lectin expressed on the surface of DCs and MΦs that recognizes mannose-type sugars on the surface of bacterial, fungal, and viral pathogens. Signals via DC-SIGN induce the phagocytosis of pathogens and contribute to host defense (54). However, DC-SIGN also interacts with carbohydrates on DENV glycoproteins and mediates the attachment of DENV to moDCs (55–57). Human immature moDCs express high levels of DC-SIGN and are highly susceptible to DENV infection (50, 58, 59). Of note,

DC-SIGN mediates virus attachment to the cell surface, but not endocytosis into moDCs (60). Activation of immature moDCs via inflammatory cytokines results in downregulation of DC-SIGN, explaining in part why mature moDCs are less susceptible to DENV infection (51, 58). Further, human cDCs freshly isolated from blood do not express DC-SIGN and, accordingly, become highly susceptible to DENV infection only after culture with GM-CSF and IL-4 that induces DC-SIGN expression (61). Similarly, treatment of monocytes with IL-4 or IL-13 increased DC-SIGN expression and DENV infection (62), but it remains unclear whether these monocytes had differentiated to moDCs or MΦs. Consistent with these findings, higher levels of DC-SIGN expression on cDCs of different blood donors correlated with higher DENV infection (61). Furthermore, a polymorphic variant of the DC-SIGN promoter with a decreased transcriptional activity correlated with protection against DF in humans (63). In summary, changes in DC-SIGN expression on different myeloid cell subsets through differentiation correlate with DENV infection (**Figure 1**).

Although *Aedes* mosquitoes transmit DENV when probing for blood vessels in the skin, most studies have focused on DENV infection in tissues after the virus has spread via the blood. Few studies have examined DENV infection and the immune response in the skin. DENV infects epidermal Langerhans cells (LCs) in healthy human skin explants *in vitro* (58, 64). Infection of LCs was confirmed in AG129 mice after intradermal inoculation of DENV2 (48). However, the dermis of intradermally inoculated *Ifnar*$^{-/-}$ mice contains 100-fold more DENV-infected cells than the epidermis (53). Recent studies indicate that dermal cDCs, and to a lesser extent MΦs, are the initial targets of DENV replication after intradermal inoculation of *Ifnar*$^{-/-}$ mice (53) or infection of skin explants from healthy human donors (65). Subsequently, *de novo*-recruited monocytes differentiate into moDCs, which become primary targets for DENV replication in the dermis (53).

Regarding the source of infectious virus, DENV produced in mosquito cells interacts with DC-SIGN and infected human immature moDCs *in vitro* (66). In contrast, DENV that was produced in human moDCs did not bind to DC-SIGN or infect moDCs but instead was infectious for cells expressing the homolog L-SIGN, such as monocytes and endothelial cells (66). The difference in binding to DC-SIGN or L-SIGN was likely due to different N-linked glycosylation patterns present on DENV particles produced in mosquito or mammalian cells. This may explain how DCs in the skin are the initial targets for DENV infection immediately after transmission. Characterization of the initial targets and immune response to DENV in the skin may foster new strategies to block DENV replication and abort pathogenesis.

ANTIBODY-ENHANCED DENV INFECTION

Dengue virus-specific adaptive immune responses, in particular subneutralizing concentrations of antibodies, can enhance DENV

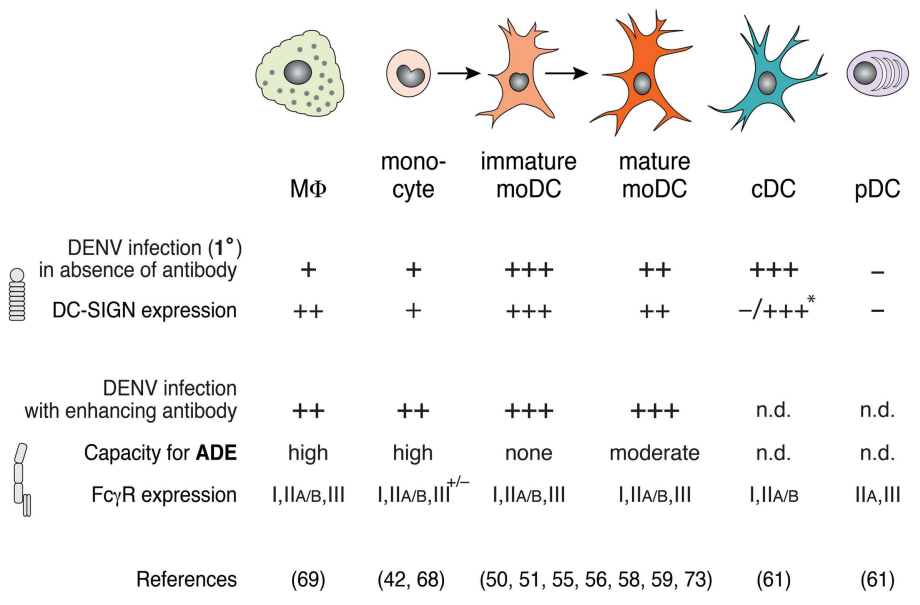

FIGURE 1 | DENV infection varies among macrophages, monocyte, and dendritic cell (DC) subsets in the presence or absence of enhancing antibodies. DC-SIGN expression correlates with high infection in the absence of enhancing antibodies (i.e., 1° infection conditions), whereas FcγR expression modulates antibody-enhanced infection during ADE. Macrophages (MΦ) and monocytes express low levels of DC-SIGN, and show little DENV infection in the absence of enhancing antibody, but are highly infected in the presence of enhancing antibody. Under inflammatory conditions, monocytes differentiate to immature monocyte-derived DCs (moDCs) and, further, to mature moDCs after stimulation via PAMPs or inflammatory cytokines. While immature moDCs express high levels of DC-SIGN and can be infected with DENV in the absence of antibodies, mature moDCs express lower levels of DC-SIGN and show moderate permissiveness under these conditions. Accordingly, mature moDCs show a capacity for enhanced infection in the presence of subneutralizing anti-DENV antibodies. Classical DCs (cDCs) that are freshly isolated from human blood do not express DC-SIGN, but express high levels of DC-SIGN after stimulation with GM-CSF and IL-4 *in vitro* (*), which renders them highly susceptible to DENV infection without antibody, similar to immature moDCs. Plasmacytoid DCs (pDCs) do not express DC-SIGN or support DENV replication in the absence of antibody. cDCs and pDCs express FcγRs, but DENV infection of cDCs and pDCs during ADE has not been determined (n.d.).

entry and infection. In cell culture, subneutralizing amounts of DENV-immune human serum or monoclonal antibodies enhance infection of monocytes (67, 68) and mature moDCs, but not immature moDCs (51). Human splenic MΦs showed low levels of DENV infection at baseline *in vitro*, but at least 10-fold greater infection in the presence of enhancing concentrations of diluted DENV-immune human (69). *In vivo*, $CD14^+$ monocytes in the blood of acute dengue patients contained significantly higher levels of DENV genomic RNA in severe DHF compared to DF cases, and in secondary compared to 1° infections (70). Thus, ADE mediates efficient DENV infection of monocytes, mature moDCs, and MΦs.

FcγR expression determines the susceptibility of cells to ADE during DENV infection via uptake of virus-antibody complexes (**Figure 1**). Most myeloid cells express FcγRs, which bind the Fc region of antibodies and thus are an important link between cellular effector functions and antigen recognition via the antibody. Different types of FcγRs recognize distinct isotypes of IgG with varying affinity and can transmit activating or inhibitory signals to the cells (71). Attachment of DENV-antibody complexes to ectopically expressed FcγRI (CD64) (72) and FcγRIIA (CD32) (73) in fibroblast cell lines mediated ADE, independently of FcγR-signaling. DENV2 infection in primary human monocytes was increased 50-fold in the presence of enhancing DENV-immune human serum and depended on binding of antibody-virus complexes to FcγRI or FcγRIIA (68). Consistent with these data, blocking of FcγRIIA, but not of FcγRIIB, abrogated ADE of mature moDCs (51). Although mature and immature moDCs express similar levels of FcγRIIA, only mature moDCs sustain antibody-enhanced DENV infection because immature moDCs express high levels of DC-SIGN and thus do not require FcγR for DENV attachment or entry (51). Inflammation may lead to activation and differentiation of monocytes and moDCs, as well as altered expression of FcγRs and DC-SIGN, which modulates ADE of DENV infection in a cell-type specific manner.

Different classes of FcγRs transmit activating or inhibitory signals and play different roles during ADE. Although activating FcγRIIA and inhibitory FcγRIIB similarly bind DENV-antibody complexes, only FcγRIIA mediates enhanced DENV infection (74). In contrast to activating FcγRs, the inhibitory FcγRIIB may help prevent ADE. DENV-antibody aggregates cross-linked the inhibitory low-affinity FcγRIIB, which inhibited the phagocytosis and infection that would have occurred through activating FcγRs (75). This study showed evidence that the size of antibody-DENV aggregates may contribute to the neutralizing versus enhancing capacity of DENV-immune sera. More detailed studies on the expression of activating and inhibitory FcγRs are needed to understand the neutralization or enhancement of DENV infection of diverse cell subsets in different tissues. Along with greater infection *in vitro*, ADE may contribute to severe dengue disease in humans. Secondary DENV infection with a heterologous serotype is associated with an increased risk of DHF/DSS (76–79). Consistent with a possible role for ADE *in vivo*, polymorphisms in FcγR genes affect binding affinities for IgG subclasses and may influence the susceptibility to severe disease. Homozygotes for the arginine variant at position 131 (R/R131) of the FcRIIA gene, who have less capacity to opsonize IgG_2 antibodies, showed reduced risk of developing DHF (80, 81). In contrast, the histidine variant H/H131 of FcRIIA was associated with an increased risk of developing DHF (81). Binding affinity of FcγRs to DENV-antibody complexes and the ratio of activating and inhibitory receptors likely determine DENV infection and disease outcome in the setting of pre-existing anti-DENV antibody.

Animal models also have been used to study ADE *in vivo*. AG129 mice develop mild disease after intravenous or intradermal inoculation with DENV2 in the absence of pre-existing antibody and lethal disease after passive transfer of subneutralizing levels of DENV-immune mouse or human serum prior to infection with otherwise sublethal doses of DENV (53, 82–84). The vascular leakage syndrome that develops in DENV-infected AG129 mice during ADE is similar to that observed after high-dose lethal DENV infection and recapitulates many features of severe dengue disease in humans (40, 44). Monoclonal antibodies directed against DENV protein E or prM can mediate ADE *in vitro* and *in vivo* (82, 83, 85). Addition of antibodies that block FcγR binding, $F(ab)'_2$ fragments that lack the Fc domain, or recombinant monoclonal antibodies that lack the ability to bind FcγR all prevented ADE (82, 83). DENV-specific monoclonal antibodies with modified Fc domains that do not mediate ADE show therapeutic potential *in vivo* (85–87).

Mouse models have helped to define possible cellular targets for antibody-enhanced DENV infection. While the same cell types become DENV infected in the presence or absence of enhancing antibodies, DENV infection increases during ADE (82). In addition, MHC II^+ cells in the intestinal lamina propria and sinusoidal endothelial cells in the liver of AG129 mice were infected by DENV mostly in the presence of enhancing concentrations of antibody (83). More detailed studies using human cells *in vitro*, clinical samples, and animal models are needed to clarify how cellular activation and differentiation modulate FcγR expression and impacts DENV infection and pathogenesis.

INNATE FUNCTION OF DENDRITIC CELLS AND EVASION BY DENV

As first line of defense against virus infection, host cells recognize PAMPs (e.g., viral nucleic acids) and induce cell-intrinsic and cell-extrinsic innate immune responses. DENV infection stimulates responses via TLR7, TLR3, MDA5, and RIG-I (88–90) and induces the secretion of IFN-α/β that renders other host cells resistant to subsequent DENV infection (91, 92).

Plasmacytoid DCs recognize DENV via TLR7 in endocytic vesicles (61, 89), become activated, produce high amounts of IFN-α (93, 94), and may thus limit DENV replication. Further, pDCs sense DENV-infected cells by direct cell-to-cell contact, and immature DENV particles containing uncleaved prM were found to trigger higher IFN responses in pDCs compared to mature particles (95). Nevertheless, DENV does not infect human pDCs efficiently *in vitro*, and IFN-α production in pDCs appears independent of active viral replication within pDCs (61). This suggests that pDCs combat DENV without being susceptible to infection or to immune evasion mediated via cytosolic viral proteins. Because DENV cannot infect pDCs productively, its proteins cannot block the production of pDC-derived IFN-α that promotes transcription of IFN-stimulated genes (ISGs), which induce an antiviral

state in neighboring cells. Patients with non-severe DF produced high levels of IFN-α in the serum (93, 96) and had an increased frequency of circulating pDCs compared to steady state (94). In contrast, numbers of pDCs declined early (day 3 or 4 of illness) in the blood of children who subsequently developed DHF (94) and, correspondingly, accumulated less in severe compared to non-severe adult cases (93). Furthermore, serum of patients with severe dengue contained less IFN-α than patients with non-severe manifestations (96). Consistent with this observation, peripheral blood mononuclear cells of patients that subsequently developed DSS expressed fewer transcripts of a set of ISGs than patients with mild disease (97, 98). Overall, pDCs protect against DENV pathogenesis by producing high amounts of IFN-α that prevent infection of additional target cells.

Dengue virus actively blocks the production and action of IFN-α/β in cell types that are susceptible to infection. The DENV non-structural protein NS2B/3 cleaves the human protein STING (also known as MITA) (99, 100), which is a key adaptor molecule in the cellular response to virus infection and in establishing the basal set-point of IRF3 signaling and IFN-α/β production (101). DENV NS5 protein induces targeted degradation of STAT2 via the proteasome (102–105) and NS4B blocks STAT1 activation (102, 106) and thus inhibits IFN-α/β and likely IFN-γ and IFN-λ receptor signaling in DENV-infected cells. However, non-infected cells remain capable of IFN production and IFNAR signaling, which may induce resistance to subsequent DENV infection. Indeed, pre-treatment of cells with IFN-α/β or IFN-γ prevents DENV infection (107–109).

Antibody-enhanced DENV infection also is believed to contribute to the evasion of the innate immune response through a mechanism termed "intrinsic ADE" (110). A monocytic cell line, THP-1, showed decreased production of inflammatory cytokines and mediators, such as IL-12, IFN-γ, TNFα, and nitric oxide radicals when they were infected with DENV in the presence but not in the absence of enhancing antibodies (111). Similarly, ADE infection suppressed TLR-mediated signals and the secretion of IFN-β, but increased the production of anti-inflammatory cytokines, such as IL-10, compared to DENV infection without antibodies (112). These effects depended on FcγR binding because antibodies blocking FcγRI or FcγRIIA restored IFN-β production (112). These findings were unexpected because activating FcγR signals should induce expression of ISGs and thus block DENV replication. Recent studies showed that the leukocyte immunoglobulin-like receptor-B1 binds DENV-antibody aggregates and blocks activating FcγR signals, which enabled DENV to evade the early antiviral response during ADE (113). These results suggest that antibody-mediated DENV entry also triggers intracellular signals that suppress innate responses in infected cells to increase viral production. This "intrinsic ADE" is complemented by "extrinsic ADE," which refers to the enhanced FcγR-mediated binding and uptake of DENV described above.

PRIMING AND EVASION OF ADAPTIVE IMMUNE RESPONSES

Dendritic cells link innate and adaptive immune responses by integrating innate signals from PAMPs with pathogen-derived antigens to induce antigen-specific T-cell and B-cell responses. DCs achieve this by (a) taking up and processing antigen and presenting antigen-derived peptides on MHC I to $CD8^+$ T-cells or on MHC II to $CD4^+$ T-cells; (b) expressing co-stimulatory molecules that activate T-cells; and (c) secreting chemokines and cytokines that attract T-cells and modulate the priming T-cell effector functions.

Because DC survival is required for optimal T-cell activation, DENV-induced apoptosis of DCs could antagonize the priming of immune responses. Bulk culture studies observed increased survival of moDCs (50, 114) and monocytes (115) after exposure to DENV. However, intracellular staining studies revealed a higher fraction of Annexin V^+ apoptotic cells in those co-staining for DENV E protein (116). These results suggest that DENV induces apoptosis in infected cells, but increases survival in non-infected bystander cells. The impact of cell survival on the number of antigen-presenting cells and priming of DENV-specific adaptive immune responses warrants further study.

Pathogen recognition leads to DC maturation, which is characterized by increased expression of MHC II and co-stimulatory markers required for efficient priming of T-cell responses (117). After exposure to DENV, non-infected bystander moDCs upregulate MHC I and II molecules, as well as co-stimulatory molecules CD80 (B7-1), CD83, and CD86 (B7-2), although DENV blocks activation and maturation of infected moDCs within the same cultures (59, 116) (**Figure 2A**). Intracellular staining for DENV proteins revealed a block in activation of DENV-infected moDCs that was not observed in bulk culture (50, 59). Similarly, non-infected bystander monocytes, moDCs and cDCs, expressed higher levels of CD80 and CD86 than DENV-infected cells in the dermis of intradermally infected *Ifnar*$^{-/-}$ mice (53). In addition, DCs produce cytokines and chemokines to modulate T-cell responses. DENV-exposed moDCs (50, 59, 116) or cDCs (61), produce IL-6, IL-10, TNFα, and IFN-α. Furthermore, DENV-exposed moDCs secrete CXCL9, CXCL10, and CXCL11 (118) that bind the chemokine receptor CXCR3 and could attract effector and memory T-cells. However, it remains unclear whether it is the DENV-infected or non-infected DCs that produce these inflammatory mediators. Together, these data suggest that DENV blocks activation in infected DCs, which may decrease the priming of $CD4^+$ or $CD8^+$ T-cells, whereas non-infected bystander cells still can become activated.

Mixed lymphocyte reactions (MLR) are a functional read-out for DC–T-cell interactions, in which DCs from MHC mismatched donors activate allogeneic T-cells. DENV infection decreased the capacity of moDCs (116, 119) as well as DCs isolated from human skin explants (65) to stimulate proliferation of DENV-naïve $CD4^+$ T-cells in MLRs *in vitro*, suggesting that DENV-infected DCs are less capable of activating $CD4^+$ T-cells (**Figure 2B**). Others have reported that DENV-infected cultures of moDCs can prime $CD4^+$ T-cells, but with decreased T-cell effector functions, such as secretion of IFN-γ or TNFα (109). The impaired ability of DENV-infected moDCs to produce IFN-α and IFN-β may explain the decreased ability to prime T-cell responses (120). Although these surrogate MLR assays are interesting, little information exists as to how DCs prime DENV-specific T-cells. Non-infected moDCs that were pulsed with DENV E protein efficiently activated $CD4^+$ or $CD8^+$ $CD45RO^+$ memory T-cells from DENV-immune but not from naïve individuals to produce IFN-γ (121). More study

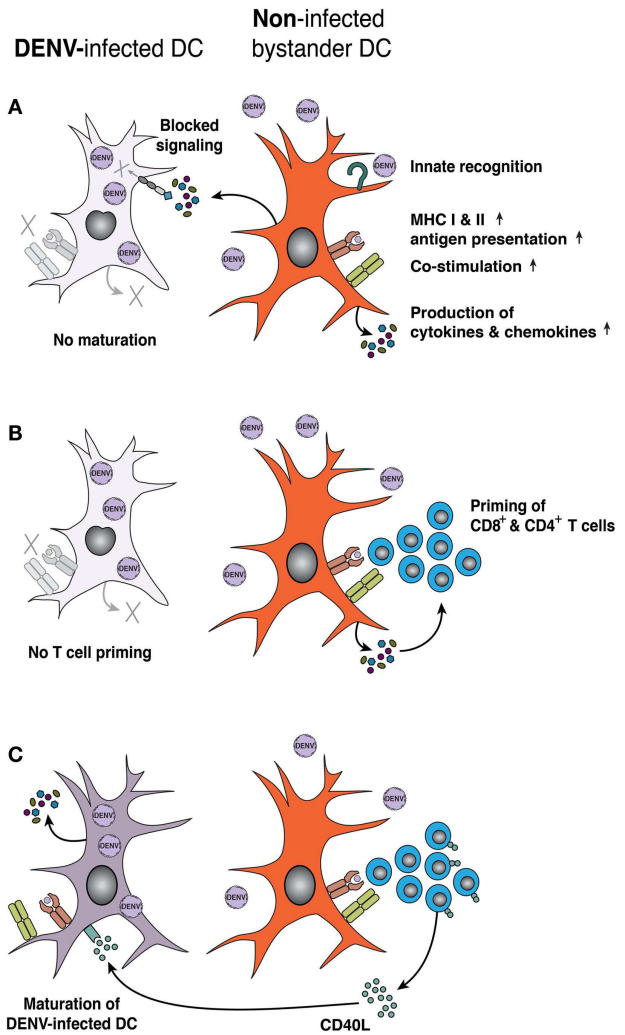

FIGURE 2 | DENV infection impairs DC activation and priming of adaptive T-cell responses. **(A)** Maturation of human moDCs is inhibited by DENV infection, due to DENV non-structural proteins blocking induction and intracellular signaling of IFN-α/β. However, non-infected bystander DCs respond to PAMPs and/or cytokines associated with DENV infection and upregulate MHC class I and II molecules, co-stimulatory molecules, and the expression of inflammatory cytokines and chemokines. **(B)** Mature, bystander DCs efficiently prime adaptive T-cell responses, whereas DENV-infected DCs prime naïve T-cells less efficiently. **(C)** Activated T-cells display CD40L on their surface or secrete CD40L that acts on DENV-infected DCs and can restore DC maturation and function. Purple denotes DENV infection, while orange denotes inflammation/cell activation.

is needed to determine the capacity of DCs to prime DENV-specific naïve T-cells or to reactivate memory T-cells during re-exposure. Rapid progress should be possible given the publication of large numbers of HLA-restricted immunodominant DENV antigens (29, 31).

Also, activated T-cells can support the maturation of DCs. An initial study showed that co-culture of moDCs with a CD40L-transfected cell line restored the ability of DENV-infected moDCs to induce MLR responses (114) (**Figure 2C**). Subsequent work demonstrated that co-culture of activated T-cells with DENV-infected moDCs rescued the otherwise suppressed MLR response, and the activation of non-infected bystander moDCs depended on TNFα and IFN-α/β (118). Consequently, stimulation of the adaptive response to DENV infection requires signals from both cells in DC–T-cell interactions, likely via cell-to-cell contact. In clinical studies, gene expression analysis of acute dengue patients revealed that DHF cases expressed lower levels of genes linked to antigen processing, presentation, and T-cell activation compared to DF patients (122). Thus, impaired antigen presentation and functionality of DENV-infected DCs may reflect a viral immune escape strategy to dampen T-cell responses and impact disease severity.

To date, mostly *in vitro*-generated human moDCs have been used to study the role of DCs in inducing innate or adaptive immune responses during DENV infection. Nevertheless, diverse subsets of cDCs in lymphoid or non-lymphoid tissues execute specialized functions in presenting antigen and inducing $CD4^+$ or $CD8^+$ T-cell responses (123, 124). Expanding previous findings using human cDC subsets directly isolated from blood or tissues will be important to characterize the full spectrum of immune responses to DENV infection.

CONCLUDING REMARKS

Dengue virus infects the same cells (DCs, monocytes, and MΦs) that are essential for inducing and maintaining optimal innate and adaptive immune responses. This tropism of DENV appears to impair DC function, which may undermine the priming of DENV-specific memory responses. Is it possible to block DENV replication in DCs to reduce viral load and restore DC function, which could impact the generation of neutralizing or potentially harmful memory B- and T-cell responses? Can we as a field harness the knowledge gained about DC biology during DENV infection to prevent human disease? Can DC function be optimized in the context of live-attenuated DENV vaccines to stimulate protective immunity?

Significant progress has been made on characterizing DENV infection and activation of DCs, as well as protective or enhancing T-cell and B-cell responses. Nevertheless, early events after DENV transmission in the skin require further study, including a greater understanding of early virus replication, local immune responses at the site of transmission (i.e., the skin), and immunomodulatory effects of mosquito saliva. Further effort should focus on how responses of DCs impact disease outcome in an acute infection and prime immunological memory responses that will affect dengue pathogenesis and disease severity in secondary DENV infections. A collaborative effort using a multi-disciplinary approach among experts in dengue virology, medicine, vector biology, and immunology is called for to reach these goals.

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Utility, limitations, and future of non-human primates for dengue research and vaccine development

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Dengue is considered the most important emerging, human arboviruses, with worldwide distribution in the tropics. Unfortunately, there are no licensed dengue vaccines available or specific anti-viral drugs. The development of a dengue vaccine faces unique challenges. The four serotypes co-circulate in endemic areas, and pre-existing immunity to one serotype does not protect against infection with other serotypes, and actually may enhance severity of disease. One foremost constraint to test the efficacy of a dengue vaccine is the lack of an animal model that adequately recapitulates the clinical manifestations of a dengue infection in humans. In spite of this limitation, non-human primates (NHP) are considered the best available animal model to evaluate dengue vaccine candidates due to their genetic relatedness to humans and their ability to develop a viremia upon infection and a robust immune response similar to that in humans. Therefore, most dengue vaccines candidates are tested in primates before going into clinical trials. In this article, we present a comprehensive review of published studies on dengue vaccine evaluations using the NHP model, and discuss critical parameters affecting the usefulness of the model. In the light of recent clinical data, we assess the ability of the NHP model to predict immunological parameters of vaccine performances in humans and discuss parameters that should be further examined as potential correlates of protection. Finally, we propose some guidelines toward a more standardized use of the model to maximize its usefulness and to better compare the performance of vaccine candidates from different research groups.

Keywords: dengue, macaques, non-human primates, vaccine, genetic, neutralizing antibodies, cell-mediated immunity, animal model

CURRENT STATE OF THE NHP MODEL TO EVALUATE DENGUE VACCINE CANDIDATES, USEFULNESS AND LIMITATIONS

Non-human primates (NHP) have been used to model a number of human infections and diseases, based on their genetic, physiological, and immune similarities with humans. In the case of dengue, NHP are the only natural vertebrate hosts besides humans susceptible to infection with the four serotypes of dengue viruses (DENV1–4). A number of species of NHP are infected in the wild by sylvatic DENV strains and experimentally by human clinical isolates without the need for virus adaptation [reviewed in Ref. (1, 2)]. A major limitation of this model for the study of dengue pathogenesis and vaccine efficacy evaluations is that the outcome of infection is subclinical in most instances (3), and does not recapitulate the symptoms seen in patients with dengue fever (DF) and dengue hemorrhagic fever and shock syndrome (DHF/DSS). Nevertheless, important similarities make it the best available model to evaluate vaccine immunogenicity and protective efficacy. Virus replication in NHP results in a peripheral viremia, with similar onset and duration to that reported in humans, although lower in magnitude. The induction of a robust neutralizing antibody (NAb) response and cellular immune responses also parallels the human immune response to dengue infection.

Two comprehensive reviews have been published recently on NHP as a potential model for dengue pathogenesis (2) and NHP infected with dengue in natural settings (1). Therefore, those topics will not be included here.

To date, in the absence of defined correlates of protection, the induction of NAbs and a significant reduction of post-challenge viremia in monkeys have been considered the closest predictors of vaccine immunogenicity and protective efficacy in humans. Therefore, the NHP model has been used for screening vaccine candidates, optimizing immunization strategies, and selecting the candidates with the best potential to work in clinical trials (4–7). Here, we present a comprehensive review of published studies on dengue vaccine evaluations using the NHP model (Table S1 in Supplementary Material). These include live-attenuated virus vaccines (LAV) attenuated by chimerization with yellow fever virus (8–15), by 3′ non-coding region (NCR) mutations (16–22), by chimerization with attenuated DENV (23–26), by serial passages in cell culture (27–29), by host range mutations (30, 31), or by mutations of a viral enzyme (32). Other vaccine platforms tested in NHP are DNA vaccines (33–39), inactivated virus vaccines (40–42), viral vectored vaccines (43–46), subunit protein vaccines (47–55), and prime/boost platforms (42, 43, 56, 57). A number of vaccine candidates tested in NHP have now been evaluated in humans, and data

are available for safety and immunogenicity in phase I/II clinical trials (4, 19, 58–79), and efficacy for the leading vaccine candidate, Sanofi's CYD1–4 (80, 81) (**Table 1**). In the light of clinical data, here we reassess and discuss the current state of the NHP model, its usefulness, limitations, and its predictive value, suggesting guidelines for the improvement of the model and the development of new reagents and standardized protocols.

EVALUATION OF ATTENUATION AND DOWN-SELECTION OF MONOVALENT COMPONENTS

One of the challenges of developing LAV vaccines is that each serotype component must be sufficiently attenuated relative to the parental strain, while maintaining adequate immunogenicity (NAbs). NHP have been used successfully to screen live-attenuated monovalent (MV) vaccine components for a balance between attenuation and immunogenicity. For example, Eckels et al. evaluated vaccine strains at different stages of attenuation by serial passages in PDK cells by screening for reduced viremia compared to parental strains while conserving immunogenicity (27). Down-selection from a number of starting candidates was achieved, and infectivity in NHP was useful to predict human reactogenicity and infectivity (82). Similarly, developers of a recombinant live attenuated dengue vaccine at the National Institutes of Allergy and Infectious Diseases (NIAID) used the NHP model to screen many MV live vaccine candidates attenuated by engineering specific attenuating deletions in the 3′NCR and/or by chimerization, ruling out several candidates that were either under- or over-attenuated, and identifying those with the most favorable attenuation and immunogenicity profile for further evaluation in clinical trials (4, 17, 18). Two recombinant DENV2 live-attenuated vaccine candidates (NIAID) with different degrees of attenuation have been characterized in both rhesus monkeys and humans for attenuation and NAb titers. The replication and immunogenicity patterns seen in NHP mimic closely those in humans (63), with one of the strains showing higher percentage of subjects with viremia and higher mean viremia titers (under-attenuated strain) in both species.

Although this model establishes general safety of dengue vaccine candidates, and can predict attenuation based on relative replication and viremia, the absence of human like symptoms limits its ability to show dengue-specific safety.

A number of non-replicating (based on DNA, inactivated virus, or subunit protein) and non-propagating (based on virus vectors) dengue vaccines candidates also have been tested in NHP for selection of antigens and antigen doses with best immunogenicity, and for the comparison and selection of vaccine adjuvants (Table S1 in Supplementary Material).

RELATIVE IMMUNOGENICITY AND SEROTYPE INTERFERENCE IN TETRAVALENT FORMULATIONS

Another challenge for the development of dengue vaccines is the need to induce equivalent and long-lasting immunity to all

Table 1 | Dengue vaccine candidates in clinical development: NHP and clinical studies.

Vaccine type	Vaccine developer(s)	Pre-clinical studies in NHP		Clinical studies		
		Study type	Reference	Study type	Reference	Current Status
Live attenuated	WRAIR/GSK	MV (S, I) TV (S, I, P) TV (I, P)	(27) (29) (28)	MV (S, I) phase I TV (S, I) phase I MV, TV (S, I) phase I TV (S, I) phase I/II	(59, 60, 69, 82) (66, 79) (78) (77)	Not being tested
	Acambis/Sanofi Pasteur	MV (S, I, P) MV, TV (S, I) TV (S, I, P) MV, TV (I) TV (I)	(13) (10, 11) (12) (14) (8)	TV (S, I) phase I TV (S, I) phase II TV (S, I, E) phase IIb (S, I, E) phase III	(68, 74, 76) (61, 70, 71) (81) (80)	In phase III
	NIAID, NIH/Merck	MV (S, I) MV (S) TV (S, I, P) MV (S, I, P)	(21, 22) (19) (16) (17, 18)	MV (S, I) phase I TV (S, I) phase I	(4, 19, 63–65) (62)	In phase II
	CDC/Inviragen/Takeda	TV (S, I, P) TV (S, I, P)	(26) (23)	TV (S, I) phase I	(75)	In phase II
Inactivated virus	WRAIR/GSK	MV (I, P)	(41, 42)			In phase I
Subunit (rE)	Hawaii Biotech/Merck	MV, TV (I, P)	(51)			In phase I
DNA	NMRC	MV (I, P) TV (I, P)	(33, 34, 36, 38, 39) (35)	MV (S, I) phase I	(58)	In phase I

MV, monovalent; TV, tetravalent; S, safety; I, immunogenicity; P, protection; E, efficacy in humans; WRAIR, Walter Reed Army Institute of Research; GSK, GlaxoSmithKline; NIAID, National Institutes of Allergy and Infectious Diseases; NIH, National Institutes of Health; CDC, Center for Disease Control; NMRC, Naval Medical Research Center.

four serotypes, due to the theoretical enhanced risk of severe disease if incomplete immunity is induced. The NHP model has been used to study serotype interference and relative immunogenicity of tetravalent (TV) vaccine components, guiding the identification of more balanced formulations and immunization strategies to minimize interference. In several instances, relative serotype dominance seen in NHP has been predictive of how the vaccine performs in the clinics, suggesting the potential for this model to predict when a candidate vaccine will induce unbalanced immunogenicity before going to clinical trials. Some examples are presented below. Guy et al. examined interference among the four serotypes of the CYD vaccine when present in equal concentrations (TV-5555) within the TV formulation in cynomolgus macaques (14) (Table S1 in Supplementary Material). NAb induced by each MV component was compared to that in the TV formulation. Interference was identified after the first and second immunizations, being serotype 4 the dominant and serotype 2 the weakest. Although each MV vaccine was immunogenic after one dose, the hierarchy of NAb titers observed for the MV vaccines was amplified in the TV mix (DENV4 > DENV1 > DENV3 > DENV2). Two phase I clinical studies in naïve human volunteers vaccinated with TV-CYD show dominance of serotype 4 as shown in NHP, while DENV1, a close second in NHP, induced the lowest titers in humans (74, 76). Monkeys receiving a second dose of CYD1–4 several months after the first dose developed a >fourfold anamnestic response to DENV1, 2, and 3, while NAbs to DENV4 were not boosted (14). Similarly, humans that received a second dose of the same vaccine CYD1–4 six months after the first one, produced anamnestic NAbs to DENV1, 2, and 3 but not to DENV4 (74), suggesting in both cases a robust sterilizing immunity to the dominant serotype 4 component. Potential approaches to minimize serotype interference were identified in macaques, by either separating the delivery of the four components in time and in anatomical sites of injection, or by adjusting the doses of each component (14). However, the lack of protection data from this study has limited its utility.

Serotype interference was also identified in different formulations of the LAV DENVax developed by CDC/Inviragen/Takeda when tested in cynomolgus macaques. When the components were present at the same concentrations, either low (TV-3333) or high (TV-5555), serotype 2 was the strongest and serotype 4 was the least immunogenic after two immunizations. By adjusting the relative concentrations (TV-3355), the immunogenicity of DENV4 was improved (26) (Table S1 in Supplementary Material). Results from ongoing phase I clinical trials (high and low doses) indicate that NAb levels against DENV2 are the highest and NAb to DENV4 are the lowest of the four serotypes in humans, as seen in macaques (23, 75). In a third example, the TV-3 formulation of the LAV vaccine developed by NIAID (LATV), resulted in robust NAbs to all 4 serotypes after a single immunization both in rhesus macaques (16) and when tested in flavivirus-naïve adult volunteers (62).

While these examples show some similarities between vaccine performances in NHP and humans, there are instances when the data do not agree, and it is unclear at this time whether these differences are the result of variability due to the small sample sizes, or to more fundamental differences between NHP and humans. As more data becomes available from clinical studies, these issues will hopefully become clearer.

ASSESSING IMMUNOGENICITY IN NHP

NAb responses to infection and vaccination in NHP

Although NAbs to flaviviruses, including dengue, have been considered necessary to prevent infection and/or disease, and required for vaccine efficacy (6), researchers are re-examining whether Ab is the best predictor of protection, and how to best measure antibody-mediated neutralization *in vitro*. This has been prompted by recent clinical findings where protection against DENV2 infection was not provided despite the presence of high NAb levels (80, 81).

Similarities in the humoral response after primary and secondary dengue infections between NHP and humans support the use of this model for evaluations of vaccine-induced Ab responses. Like humans with primary dengue infection or immunized with a MV vaccine, a number of NHP species can mount robust long-lasting serotype-specific NAbs responses that prevent reinfection with the same serotype, and short-lived cross-protective NAbs (83–88). Koraka et al. showed that the level and duration of viremia, and the kinetics and magnitude of Ab responses observed in experimentally infected macaques were similar to those observed in most uncomplicated human dengue infections, and that Abs measured were largely cross-reactive (88). Upon secondary infection, or immunization with multivalent vaccines, NHP show a broad NAb response to multiple serotypes (88). Secondary infection with DENV3 following a DENV1 infection suggested the phenomenon of original antigenic sin (89) in macaques for that sequence of infections (88).

Recent studies point to potential similarities in the quality of the induced NAbs in NHP and humans. In-depth characterization of the primary response in NHP reveals that unlike mice but similar to humans (90), NHP produce serotype-specific NAbs that predominantly bind to sites other than domain III on the E glycoprotein (EDIII) (46). Interestingly, there is evidence that the nature of the DENV antigen modulates the NAb targeting. For example, soluble E ectodomain expressed using an alphavirus-vectored vaccine (VRP) induces in NHP NAbs that bind predominantly to EDIII, while VRP expressing prME subviral particles induce in NHP predominantly non-EDIII binding antibodies (46). A recent study on epitope targeting using an epitope transplantation approach showed that the E domain I/II hinge region of DENV3 and DENV4 is the primary target of long-term, serotype-specific NAbs in humans and in rhesus macaques after primary infection (91). One implication of these observations is that the quality of the NAb response in NHP and humans may be different between vaccines based on live-attenuated virus or inactivated virions and protein subunit vaccines, and the NHP model may be able to predict these specificities. These different vaccine platforms need to be tested in NHP and in humans in order to help answer the question of what region(s) on E protein are targeted by NAbs induced by a successful dengue vaccine.

The genotypic breadth of vaccine-induced NAb responses is an important Ab quality to examine, since some vaccine antigens may have a narrower set of serotype-specific epitopes that may affect the

breadth of the protective immunity. Recent studies have addressed the breadth of vaccine-induced NAbs in NHP (8, 16, 46).

NAbs and protection from viremia in NHP

The ability of NAbs to mediate protection in NHP has been shown by passive transfer experiments (92, 93). Hahn et al. showed that the infusion of a bivalent monoclonal antibody in NHP was followed by subsequent clearance of dengue virus from the vascular system (92). Lai et al. demonstrated protection against DENV4 challenge in rhesus monkeys by passively transferred humanized monoclonal antibody (93). In most vaccine studies in NHP, the presence of pre-challenge NAbs seems to correlate with reduction or absence of post-challenge viremia. However, a threshold titer that correlates with protection in NHP has not been identified, and there are reports of animals with moderate to high $Neut_{50}$ titers that show breakthrough viremia (12, 26, 29, 45, 46). To determine whether combining and analyzing data from several studies would provide additional insight, we collected data from 10 published studies of dengue vaccine candidates in NHP, and used them to graph pre-challenge $Neut_{50}$ titers vs. duration of viremia (**Figure 1**). Four graphs were generated, **Figures 1A–D**, corresponding to protection from viremia after challenges with dengue serotypes 1–4, respectively. Only studies that reported $Neut_{50}$ titers and duration of viremia for individual monkeys were included, and for each study, data from both immunized and unimmunized controls were used (12, 26, 29, 40, 43–46, 49, 51). A strong negative correlation between pre-challenge $Neut_{50}$ titers and duration of viremia was observed, with Pearson correlation coefficients (r) of −0.4453, −0.3367, −0.4182, and −0.3063 for serotypes 1–4 challenges, respectively. A high percentage of animals with titers = or >20 had no viremic days after challenge (77% for DENV1, 76% for DENV2, 94% for DENV3, and 72% for DENV4 challenges).

Figure 1 also shows breakthrough viremia for some animals with $Neut_{50}$ titers >20, although in most cases the viremia was of shorter duration. The number of monkey with viremia out of those with titers of 20 or higher were 10 out of 43 (23%) after DENV1 challenge, 10 out of 41 (24.4%) after DENV2 challenge, 2 out of 36 (5.5%) after DENV3 challenge, and 7 out of 25 (28%)

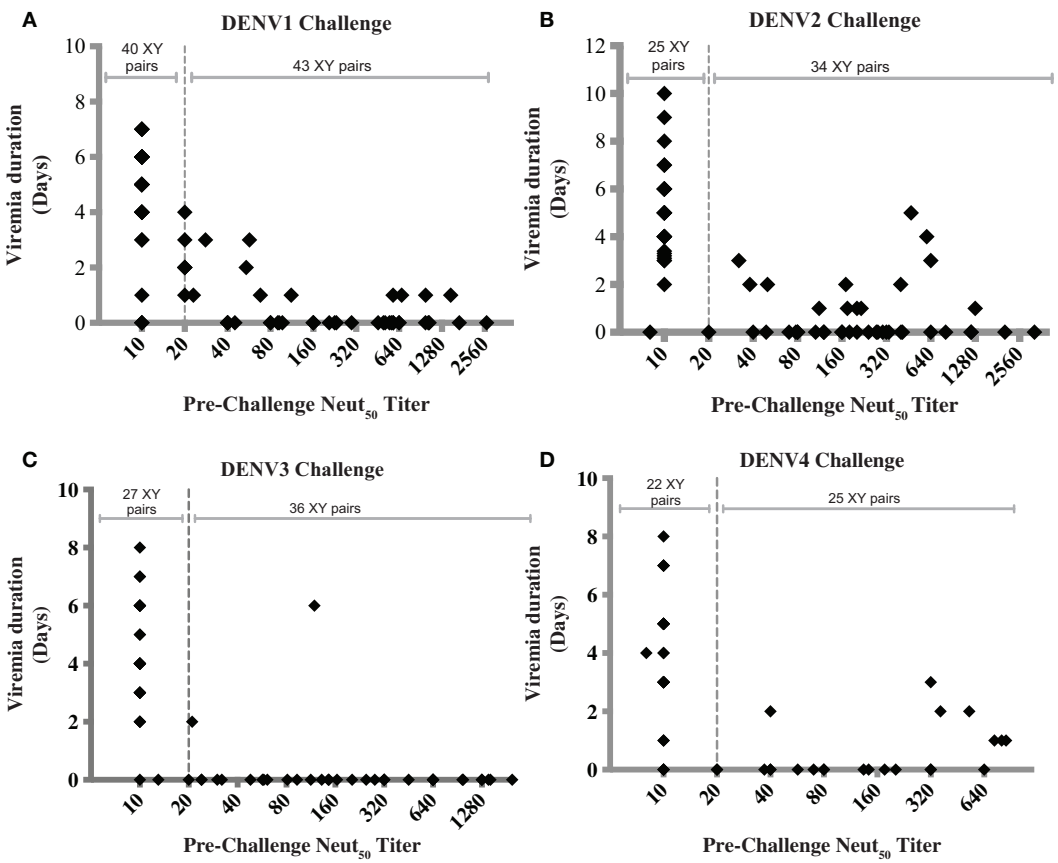

FIGURE 1 | Correlation between pre-challenge $Neut_{50}$ titers and duration of post-challenge viremia. Each data point was obtained from 1 of 10 published studies on dengue vaccine candidates tested in NHP. Only studies that reported $Neut_{50}$ titers and duration of viremia for individual monkeys were included, and for each study, data from both immunized and unimmunized controls were used [Men et al. (44), Guirakhoo et al. (12), Sun et al. (29), Chen et al. (43), Raviprakash et al. (45), Bernardo et al. (49), Clements et al. (51), Osorio et al. (26), Maves et al. (40), White et al. (46)]. Data were combined based on the challenge virus serotype into graphs **(A–D)**, corresponding to serotypes 1–4, respectively, regardless of vaccine type or whether it was monovalent or tetravalent. Dotted line indicates a $Neut_{50}$ titer of 20. Titers below the limit of detection were given a value of 10, whether the limit of detection was 10 or 20. The number of XY pairs with $Neut_{50}$ titers <20 or ≥20 are indicated at the top of each graph. Pearson correlation coefficients (*r*) were −0.4453, −0.3367, −0.4182, and −0.3063 for graphs **(A–D)**, respectively.

after DENV4 challenge. An interesting finding is that the proportion of breakthrough viremia cases after DENV3 challenge was lower (5.5%) compared to the other three serotypes, suggesting that a surrogate of protection may be serotype-specific.

The interpretation of these combined data is limited by the use of a single readout of protection, duration of viremia, and because different methods to measure viremia were employed. In addition, differences in the challenge strains and doses probably added variability to the data, which is evident in the broad range of duration of viremia in unvaccinated controls (1–8 days). Also, the data includes MV and TV vaccine studies. In the case of TV vaccine studies, there are differences in the time between the last immunization and the challenge, which may have affected the levels of short-lived heterotypic, potentially protective NAbs at the time of challenge. However, only homologous $Neut_{50}$ titers are used in the analysis.

In spite of these caveats, examining the collective data in this way suggests a strong correlation between NAb titers and protection in the NHP model, but also indicate that NAb titers >20, as measured *in vitro* on epithelial cells, not always prevent challenge virus replication in the NHP model. This analysis raises interesting questions. For example, could some cases of breakthrough viremia be explained by the induction of a qualitatively distinct group of NAbs that can neutralize infection of epithelial cells *in vitro* but not infection of target cells *in vivo*?

On the other hand, protection from viremia has been shown in rhesus macaques with poor NAbs (41, 94) or after NAbs have waned (39), suggesting that other immune mechanisms may play a role in protection, especially when Abs are suboptimal. This is supported by studies in mice, where cellular responses were sufficient to protect from lethal challenge (95).

Current in vitro neutralization assays

Most DENV vaccine pre-clinical and clinical studies reported to date examine neutralizing activity in serum by measuring *in vitro* neutralization on epithelial cells of animal origin (LLC-MK, BHK-21, Vero), using the standard dengue PRNT assay, originally described by Russell (96), and recommended by the World Health Organization (WHO) (97) (Table S1 in Supplementary Material). Although PRNT is considered the gold standard, its use is not standardized among labs, and protocol variations in cell lines, PRNT end point titers, virus passage number, and presence of complement are known to affect the Ab titer readout (98, 99). Variations of the PRNT have been developed for higher throughput, reduced duration and labor, to test against DENV clinical isolates that do not plaque well, and to measure neutralization in more biologically relevant cells, like primary human myeloid cells. Alternative assays include ELISA-based microneutralization (ELISA-MN) (100, 101), flow cytometry-based assay using Vero cells, or DC-like cells (46, 102, 103), assays based on FcγR-bearing human cells (104–106), and a reporter virus based system (107). A comparative evaluation of MN and DC assays vs. PRNT indicated that the assays are not always in agreement (106). Recent studies show that NAbs measured on epithelial cells result in different titers compared to assays that use FcγR-bearing cells (105, 108, 109).

There is an urgent need for determining what *in vitro* neutralization assay(s) best correlate with protection in NHP and in humans, and to minimize assay variation and experimental inconsistencies in the *in vitro* neutralization assay, which have made it difficult to compare NAb titers among studies.

It is important to note that currently used neutralization assay cannot determine whether a TV response is generated by four serotype-specific responses or from cross-reactive short lived and less protective Abs. Therefore, only by allowing cross-reactive short-lived antibodies to decay, and confirming TV responses after 6–12 months, will the assay measure truly serotype-specific NAbs.

Cell-mediated immunity

To date, cell-mediated immunity (CMI) is not required for clinical evaluation of dengue vaccine candidates. However, after results of the first efficacy study in humans, there is increased interest in measuring CMI in vaccines tested in clinical trials due to their potential role in protection (81, 110). There have been a few studies addressing CMI in NHP after DENV infection and vaccination (Table S1 in Supplementary Material). These studies have examined the induction of dengue-specific cytokine-producing cells from PBMC stimulated with purified DENV or NS1, NS3, or NS5 peptide pools, using ELISPOT or intracellular cytokine staining (ICS) or cytotoxicity assays (23, 25, 26, 28, 35, 39, 43, 45, 50–52).

Cell-mediated immunity after primary, secondary, and tertiary experimental infection in cynomolgus macaques has been examined by Koraka et al. (88). Bulk T-cell-mediated responses were found against homologous and heterologous viruses even after primary infection. T-cell-mediated IFNγ production measured after secondary DENV3 infection following a DENV1 infection suggest a phenomenon of original antigenic sin, as described after human infections (111). Mladinich et al. (112) studied the kinetics of DENV specific T cells in rhesus macaques after primary infection with DENV2, showing multifunctional $CD4^+$ and $CD8^+$ T cells specific for NS1, NS3, and NS5. These studies suggest that infection of NHP with DENV result in CMI that are similar to those in humans in kinetics and serotype-specificities (110, 113), and suggest that NHPs may be a useful model to further understand the cellular responses to vaccine candidates and their role in pathogenesis (88, 112).

Vaccine-induced cellular responses have been studied in NHP for a few vaccine candidates. Osorio et al. (26) reported that macaques immunized with TV live-attenuated vaccine DEN-Vax showed robust numbers of DENV-2-induced IFNγ and IL-2 secreting cells by ELISPOT, when peripheral blood mononuclear cells (PBMCs) were stimulated using semi-purified concentrated wt DENV-2. Consistent cytokine-secreting cells stimulated by DENV1, DENV3, or DENV4 were not observed (26). A study by Ambuel et al., using ICS and stimulation with NS1, NS3, and NS5 peptide pools, reported that the same DENVax vaccine-induced $CD4^+$ and $CD8^+$ T cells producing IFN-γ, IL-2, and TNF-α and targeted the DENV2 NS1, NS3, and NS5 (23). Human T cell data from clinical trials is pending. A DNA vaccine developed by the Naval Medical Research Center (NMRC), D1ME100 has been tested in NHP and in phase I trials (Table S1 in Supplementary Material). Chen et al. reported dengue-specific T cell responses in cynomolgus macaques immunized with three doses of a plasmid DNA vaccine expressing DENV1 prME (43). The cellular responses were measured by stimulating PBMC from immunized

animals with purified DENV1 and measuring IFNγ secreting cells by ELISPOT. When the prototype DENV1 DNA vaccine D1ME100 was tested in humans at high and low dose using biojector2000 to deliver, T-cell IFNγ responses were detected after three immunizations in 50 and 83% of subjects in the low (1 mg) and high (5 mg) dose groups, respectively (58).

In summary, measuring CMI is complex and requires careful sample collection and storage, and standardization of assays and antigens used to stimulate immune cells. Like the NAb assays, CMI assays lack standardization of critical reagents and methods. Semi-purified DENV as stimulating antigen does not result in optimal T-cell responses. While peptide arrays for a subset of viral proteins and serotypes are now available through BEI Resources, other antigens/peptide arrays should be developed and validated. Tracking specific $CD4^+$ and $CD8^+$ T-cell responses has been difficult due to a lack of mapped epitopes and associated reagents within the macaque model. Mapping DENV specific $CD4^+$ and $CD8^+$ T-cell responses in NHP and producing MHC:peptide tetramers would greatly advance this model and accelerate the pace of dengue virus vaccine development. The availability of these reagents would allow performing more in-depth analysis of vaccine-induced $CD8^+$ T-cell responses that may be critical for the success of future dengue virus vaccines.

THE NHP DENGUE CHALLENGE MODEL

In the absence of dengue-induced disease in NHP, viremia is measured post-challenge as a surrogate of protection. Even though it is not required by the Food and Drug Administration (FDA), to date all dengue vaccine candidates in clinical trials have previously shown efficacy in NHP (114). However, results of the lack of protection by the CYD1-4 vaccine candidate in the first phase IIb efficacy trial (81), and lack of protection against serotype 2 in the first phase III efficacy trial (80) raise questions of whether the NHP challenge model is able to predict efficacy in humans. Unfortunately, the DENV challenge model in NHP is not standardized, making it difficult to compare the results from studies performed by different studies (Table S1 in Supplementary Material). We discuss below how specific limitations of the NHP challenge model may affect its performance.

Diversity in NHP used for Dengue studies

A number of NHP species used to study dengue pathogenesis and vaccines evaluations are listed in **Table 2**, along with information on the relative frequency with which they have been reported, the number of MHC alleles known and sequenced to date for each species, and an estimated number of reagents and/or protocols available through a free resource tools in support of research.

The use of multiple species makes it difficult to integrate results from different studies. In addition, within the same species, the genetic composition and variability in the genetic background of animals in the study can have an impact on the results (115). It has been well documented that the geographic region from which the founder animals are derived determines the MHC haplotype composition of the population (116, 117). Such genetic differences may also contribute to the phenotypic variance of pre-clinical trials when animals from different locations are included as experimental subjects, or when animals from different countries are compared. High inter-animal additive genetic variances increase phenotypic variance in full-breed animals and can obscure correlates under study (116, 117). Theoretically, this may be particularly relevant when addressing the role of cellular-mediated immune responses, but no data are available yet. On the other hand, as shown in Table S1 in Supplementary Material, six species of NHP have been used to study vaccine-induced immunogenicity, and NAbs have been reported in all of them. In addition, the same vaccine candidate has been tested in two species of monkey for two vaccine platforms, CYD by Sanofi (10, 14), and DNA by NMRC (34, 39), and no major differences in NAb responses can be attributed to the monkey species used. However, in most studies in this report, large animal-to-animal variation among NAbs titers exists even among animals in the same experimental group.

The more common species in use in biomedical research, rhesus macaques, can be very diverse in its genetic background, with groups of animals with Indian origin clustering more close with

Table 2 | Species of NHP used in dengue pathogenesis and vaccine studies.

Common name	Scientific name	Percent of published reports (1,178 total) (%)	MHC designation/no. alleles available[a]	Number of available resources (antibodies, cell lines, SOPs) (NIH non-human primate reagent resource)[b]
Rhesus macaques	Macaca mulata	51.35	Mamu/1,197	221
Green monkeys	*Cercopithecus aethiops*	34.80	*Chsa*/64	38
Cynomolgus macaques	*Macaca fascicularis*	11.62	Mafa/1,506	202
Patas monkeys	*Erythrocebus patas*	1.0	N/A	N/A
Yellow baboons	*Papio cynocephalus*	0.6	*Papa*/30	N/A
Japanese macaques	*Macaca fuscata*	0.33	*Mafu*/27	N/A
Mangabeys	*Cercocebus spp*	0.1	N/A	193
Others		1	N/A	N/A

[a] *Major histocompatibility complex genes of non-human primates. Available from the immuno polymorphism database (IPD), European molecular Biology Laboratory, and European Bioinformatic Institute.*

[b] *www.nhpreagents.org*

animals belonging to western China than to the other Indian group, consistent with the hypothesis that they originated in Burma, which is very close of western China (117). It has also been proposed that a natural gene flow among populations of Chinese (and Burmese) rhesus macaques and Vietnamese cynomolgus macaques (*Macaca fascicularis*) exists (115, 117, 118).

Being of intermediate genetic composition, admixed animals may respond very differently to experimentation involving traits that have a phenotypic variance that is lower than either unmixed parent population due to increased heterozygosity and stabilizing selection. The Cayo Santiago colony at the Caribbean Primate Research Center (CPRC) is an example of a population of rhesus macaques used in biomedical research in the US in which all the animals are derived from founders from the Indian subcontinent and it has been demonstrated that this population is genetically homogenous and unadmixed (117). Populations of cynomolgus macaques, the second most frequent species used for dengue studies can be even more genetically divergent as they may have originated from different locations in Southeast Asia including Sumatra, Corregidor, Mauritius, Singapore, Cambodia, Zamboanga, and others. Genetic variations among regional populations of cynomolgus macaque are the main cause of differences in research subjects that determines the repeatability of experimental outcomes (115). While the impact of the genetic diversity among and within the used species on DENV replication has not been addressed carefully, there have been reports of animals that can be considered as natural viremia controllers (29, 67, 93, 119–121).

Genetic homogeneity among research animals allows for the utilization of fewer subjects and cost-effective research projects. The origin ancestry and pedigree of the dengue research subjects should be taken in to account. It has been proposed that to maximize resolution of experimental treatment effects, animals of unmixed ancestry with paired coefficients of relationship below that of first cousins ($r < 0.125$) should be used (115). These considerations have important implications on what should be the minimum number of monkeys per experimental group. Supported by published data and in our own experience, cohorts should include at least six and not less than four, to minimize the impact of the genetic variability in the results.

DENV challenge strain, dose, and route

A comprehensive review of DENV strains used to infect different species of NHP and the viremia they caused can be found in Clark (2) and Hanley (1). Most vaccine studies in NHP include a challenge with one or more DENV serotypes to assess protective efficacy. The challenge strains used vary among studies, including wild-type viruses, near wild-type viruses or homologous viruses parental to the vaccine strains (Table S1 in Supplementary Material). To date, there is not a repository stock of DENV strains for each serotype available to different research groups, nor a standardized challenge protocol. These are in urgent need in order to guarantee the reproducibility and comparability of the results. Even using same strains by different groups has shown different outcomes. Hickey et al. (86) were unable to confirm viremia in groups of four rhesus macaques challenged with strains of dengue 1 and dengue 4 shown to induce detectable viremia in rhesus macaques in different protocols conducted by other groups (16, 46). This confirms that in addition to the virus strain, dose, and route of administration, other variables like the challenge virus passage history and the time post-challenge for sample collection, among others, need to be considered.

To determine protective efficacy, the dose of the challenge virus has to ensure a measurable and reproducible viremia for several days. Only a limited number of studies have attempted determining the infectious dose delivered during natural dengue infection. One study suggests that the amount of infectious particles transmitted by *A. albopictus* ranges from 1×10^4 to 1×10^5 plaque forming units (PFU) (122).

To our knowledge, the only study comparing inoculum dose and viremia in NHP was performed by Halstead et al. (123). In that study, animals were challenged with low (8–50 PFU) and high doses (5×10^3–5×10^5 PFU) of DENV, which resulted in levels of viremia ranging from 0.6 to 1.8×10^3 PFU/ml. In general, doses from 1×10^4 to 1×10^5 PFU, independent of the route, have shown to be enough to induce detectable viremia and seroconversion in most of the animals, or strong seroconversion in spite of absence of viremia [reviewed in Ref. (124)]. Higher doses do not seem to result in higher viremia. Even doses of 1×10^7 PFU delivered via intravenous injection, resulted in peak viremia of 8×10^3 PFU/ml (3). In addition, a significant negative relationship between challenge dose and duration of viremia has been documented (125).

When to challenge and protection readouts

The time allowed between the last immunization and the challenge varies from 30 days to 12 months in reports of dengue vaccine studies in NHP (Table S1 in Supplementary Material). These differences make it difficult to compare vaccine efficacy among vaccine candidates from different studies. There seems to be consensus in the field to allow at least 6 months between the last immunization and the challenge. This would increase the stringency of the challenge and better assess protection mediated by serotype-specific, long-term protective immunity, minimizing protection readouts mediated by short-term heterotypic immunity. In humans, heterotypic protection after primary infection lasts for a few months (126). NHP, like humans, develop long-term serotype-specific NAbs that last as long as the duration of the study, 13 (86) or 24 months (91), and shorter-lived cross-reactive NAbs. Hickey et al. reported that the duration of the heterotypic NAbs varied among serotypes and among animals; these NAbs were mostly absent after 120 days in a DENV3 infection, while decreased at different rates between 120 and 390 days in animals infected with the other serotypes (86). Messer et al. confirmed the presence of homotypic NAbs up to 24 months after infection with DENV3 or DENV4, and also showed detectable NAbs ($Neut_{50}$ titer = 35) against the heterologous serotype in one out of four animals 24 months after infection (91).

The major limitation of the NHP dengue challenge model is that protection from low-level viremia in NHP may not reflect protection from DF or DHF in humans. In the absence of better defined correlates of protection, different readouts of protection in the NHP model have been proposed: (1) prevention of infection (sterilizing immunity), measured by absence of viremia, and

absence of anamnestic response, as defined by fourfold increase in NAb titer. (2) Significant reduction in the duration and magnitude of the viremia (infectious virus or RNA genome equivalents) without preventing an anamnestic response, where a robust anamnestic response is indicative of a robust memory response with potential to prevent disease. To date, there is not clear evidence that the more stringent criteria for solid immunity in NHP is what correlates with protection in humans. Most vaccines tested in NHP show some level of anamnestic responses (Table S1 in Supplementary Material), suggesting non-sterilizing immunity. Sterilizing immunity has been reported after DENV1 MV NIH LAV vaccination and challenge (22), and after one dose of LATV followed by DENV4 challenge (16). Viremia has been measured by different methods. A semi-quantitative measurement involves amplification of the virus collected in the blood in insect or mammalian cells followed by IFA (34). Quantitative determinations of infectious virus particles are done by plaque assays and immune-focus assay (19, 26, 46, 86, 87, 91). A quantitative RT-PCR has been used to determine RNA genome equivalents (20, 32, 48, 86, 88, 121, 127, 128). However, components in the monkey sera can inhibit non-specifically the RT-PCR assay, rendering false negatives or amplifications that are difficult to interpret, even when using virus stocks that have been shown to induce productive infection in NHP measured by other methods (83, 86, 120). In addition, time to viremia was significantly shorter, and duration of viremia was significantly longer when measured by RT-PCR compared to plaque forming units (125). Based on these results, it is recommended that more than one method be used to characterize and quantify post-challenge viremia.

Toward a NHP disease model

Although most dengue infections in NHP are subclinical, cutaneous hemorrhages, and lymphadenopathy have been reported (2, 3, 123, 129–131), and only a small fraction of studies have reported rashes post infection (3, 129). Onlamoon et al. showed recently that by using an i.v. route and a high dose of 10^7 PFU, a primary infection in rhesus macaques with DENV2 strain 16681 resulted in cutaneous hemorrhages, and suggested that further amplification of disease severity could result from refining other parameters like virus strain, factors from infected mosquito saliva, and macaque genetic factors (2, 3). Although such manipulations of route and dose of infection distant the model from naturally occurring dengue, further exploring this disease model may help understand the impact of dengue infection on a set of particular cells playing a key role in pathogenesis in a higher animal model.

Another potential factor that may modulate dengue infection and the course of disease is mosquito's saliva. There are a number of studies supporting its modulatory role on the immune response to dengue virus (132–137) and other arboviruses (138–145). However, its potential role modulating disease in NHP has not been examined. Studies comparing the course of infection and immune response between viruses delivered into the skin by needle and by mosquito bite should be done. Until then, we could be missing the role of an important component defining the quality of the immune response to dengue in nature and overlooking potential key data from the pre-clinical dengue vaccine trials.

Sequential infections modeling ADE

Early work by Halstead et al. showed that after a secondary DENV infection in NHPs viremia increases, suggesting that ADE may increase viral load through cross-reactive Abs (129, 146). However, because few numbers of animals were included in each experimental group, only a trend was reported but no significant differences were established.

A cross-reactive response with the highest Ab titers directed against the primary infecting serotype (and potential ADE induction) was showed only when the sequential infection was DENV1–DENV3 but not when DENV4 was the primary infecting serotype (87, 88). *Aotus nancymae* monkeys sequentially infected with dengue 1 followed by dengue 2, either with an American or an Asian genotype, did not showed any significant viremia increase, ruling out ADE mechanism for this particular sequential infection (87). Recently, it has been showed that primary infection with serotypes 1, 2, and 4 but not serotype 3 induces long-lasting cross-reactive neutralizing antibodies in NHP. However, as these antibodies decrease after 120 days of infection, the host may become susceptible to develop ADE after DENV1, 2, or 4 but not DENV3 infection (86). The passive transfer of anti-DENV monoclonal Ab 1A5 prior to DENV infection resulted in a viremia increased 3- to 100-fold in RMs after (147). A previous study had shown that the administration of polyclonal diluted dengue 2 immune cord-blood serum to few animals resulted in increased viremia after infection with dengue 2 (148). The data reviewed here confirms that the role of ADE in NHP infection is still controversial. However, due to the impact of ADE in dengue pathogenesis (149) and its implications in the quality of the immune response elicited after vaccination, NHP should continue to be explored as a potential contributor to the understanding of the role of ADE in dengue pathogenesis.

THE FUTURE OF THE NHP MODEL

The value of the NHP model for dengue research has the potential to be of larger scope than to date.

IN-DEPTH CHARACTERIZATION OF IMMUNITY TO VACCINATION

A better characterization of the immune response to vaccination may help define better correlates of protection. These include mapping where NAbs bind, measuring early type-specific antibody secreting cells (ASC), Ab avidity, and neutralization in FcγR-expressing cells. Studies should include genotypic breadth, contribution of type-specific vs. cross-reactive antibodies, epitope repertoire, ADCC, role of complement, and cellular response, including multifunctionality of $CD8^+$ T cells. Recent studies have shown the plasticity of this model allowing the replication of chimeric virus carrying transplanted EDI–EDII hinges (91), and at the same time, confirming the value of the model for a better mapping of the antibodies repertory *in vivo* (46, 91).

MODELING VACCINATION IN DENGUE ENDEMIC REGIONS

Most dengue vaccines tested in NHP use dengue naïve animals, and in a few cases animals immune to yellow fever. Since most vaccines will be used in dengue endemic countries, studying how vaccines perform in monkeys after passive transfer of dengue Abs or after previous wild-type dengue virus infection will be of great value.

NHP MODEL VALIDATION BY CLINICAL STUDIES

The predictive value of the NHP model for candidate vaccine efficacy is limited by the lack of efficacy data available from studies of parallel vaccines in humans, lack of standardized protocols and reagents, and lack of in-depth immunological studies in primates and humans to infection and vaccination. As phase III clinical trial data becomes available, and as human challenge is incorporated into early clinical testing (150), comparison of results from ongoing clinical evaluations and NHP studies will lead to validation and improvement of the NHP model, to become a better predictor of the human response to vaccination and become a more robust model, reducing the need to do human challenges. For this to happen, results must be shared promptly among vaccine developers and the dengue research community, to expedite and facilitate the identification of better correlates of protection.

TESTING MODIFIED MAbs AS THERAPEUTICS

From therapeutic point of view, vaccines have been the dominant if not the unique approach intended in NHP to fight dengue virus infection (4–6, 46). However, other therapeutic approaches like the use of MAbs are almost unexplored. Recently, it has been shown the effectiveness of using MAbs to control dengue infection in mice, and *in vitro* methods have been developed that predict the ability of modified MAbs to act therapeutically against antibody-enhanced disease *in vivo* (151). The potential of these therapeutics tools in humans and alternatives for improvement have been extensively reviewed (152). However, few studies have been conducted in NHP. Hahn et al. showed that the infusion of a bivalent monoclonal antibody in NHP was followed by subsequent clearance of dengue virus from the vascular system (92). Passive i.v. transfer of antibody IgG 5H2ΔD protected monkeys against DENV-4 infection and this was confirmed by absence of both viremia and specific anti-dengue antibodies (93). These studies showed the feasibility of using NHP to test and to develop those new therapeutic alternatives. Furthermore, over the past 3 years, we have learned that NAb responses in mice and people target different domains on DENV particles (EDIII vs. EDI–EDII hinge) (46, 90, 153–156). Moreover, our group has confirmed that NHP exposed to natural DENV infections appear to develop neutralizing Abs responses that are qualitatively similar to the human response (46, 154). For this reason while mice continue to be a very useful model for screening, the final functional significance of therapeutic MAbs should be tested in macaques (90, 154, 155). Although they could be expensive, therapeutic MAbs would help saving lives if they are used in the first hours/days after the infection in those patients where severe manifestations can be anticipated using the WHO guidelines for dengue case classification (157).

Another alternative therapeutic approach is the use of Toll-like receptors (TLR) agonist in vaccine formulations or anti-viral strategies against different pathogens. TLR have been already assayed for other viruses (158–164). However, so far only one study addressed the impact of TLR agonists on dengue virus immune response *in vivo* in NHPs (121). This work and the fundamental role of the TLRs in modifying the immune/vaccine response guarantee further studies on the impact of TLRs in dengue pathogenesis/vaccine studies.

STANDARDIZING THE USE OF THE NHP MODEL

Based on recommendations compiled in the literature (114, 165, 166) and from discussions with colleagues in the field, the following guidelines toward standardization are proposed.

Animal selection

- Select healthy animals with a homogenous genetic background (coefficients of relationship below $r < 0.125$).
- Most published studies using four to six animals per experimental group have yielded interpretable data. However, in many cases, increased statistical rigor is needed. The minimum number of animals per group to have adequate statistical power should be calculated for each specific objective (167).

Evaluation of immunity

- Until an improved and more predictive *in vitro* neutralization assay is developed and validated, the WHO recommended that a standard plaque-reduction (PRNT) or focus-reduction neutralization assay should be used to evaluate vaccines in NHP, using optimized protocols and reagents and reference virus strains representing different serotypes and genotypes.
- Evaluation of CMI is strongly recommended as part of the analysis of vaccine immunogenicity in NHP. Standardized procedures for collecting, processing, and storing of PBMC should be used. PBMC should be collected before vaccination to establish a baseline, and then at various time points after vaccination to measure effector function, memory, and durability. Stimulating antigens should be well-characterized and available to the dengue community.
- Post-challenge NAbs should be measured, to determine whether the vaccine induces anamnestic responses and sterilizing immunity. This information will be of value once phase III clinical data is available.
- When possible, each monotypic component of the vaccine should be tested alone and combined in the TV formulation, to evaluate serotype interference.

Dengue challenge and protection

- A collection of well-characterized challenge viruses should be available to all researchers as a publicly funded repository, such as BEI resources. Researchers with strains that replicate well in NHP should contribute their strains to this collection.
- Post-challenge viremia should be determined by at least two different methods, one of them being a plaque- or foci-based assay for infectious virus. Universal techniques should be adopted.
- Challenge should be done at least 6 months after the last immunization, using homotypic wild-type virus strains that have been well characterized previously and result in sustained and consistent viremia for several days. The doses of challenge virus to obtained consistent viremia should be pre-determined, and generally ranges between 10^4 and 10^5 PFU.

ETHICS AND HUMANE USE OF NHP

For most candidate human vaccines, including those for dengue virus, immunogenicity, and if possible protective efficacy of the candidate formulation, has to be shown in a relevant animal model before it is tested in humans. There is no *in vitro* correlate of *in vivo*

immunogenicity and protective efficacy for candidate dengue vaccine formulations. Therefore, animal models must be used in the development of dengue vaccines to screen potential candidates and allow those that show robust immunogenicity to move forward into clinical trials. Most of the work reviewed here included ethical statements on the humane use of animals.

Authors strongly encourage all researchers working with NHP to follow the local regulations on the use of NHP for research. Also, we support the implementation of additional steps to ameliorate suffering in accordance with the recommendations of the Weatherall report, "The Use of Non-human Primates in Research." It is also advised to have animals under an environmental enrichment program approved by the local committee.

CONCLUSION

To date, NHP data have been the gatekeeper for vaccines advancing to clinical trials, based on neutralizing activity in serum and reduction of post-challenge viremia, which indicates to vaccine developers and regulatory agencies of the potential for efficacy in humans. Recent clinical data has become available to compare vaccine performance in NHP and humans. Such analysis indicates that the replication and immunogenicity of vaccines tested in NHP has parallels to the human responses to the same vaccines, specifically regarding under-attenuation, relative serotype dominance, and immunogenicity in TV formulations, induction or not of anamnestic responses upon second vaccine doses and seroconversion to all four serotypes after a single dose in most subjects. These results suggest that a more comprehensive study of the immune responses to infection and vaccination in NHP may significantly help identify new immune correlates of protection.

Until molecular correlates of heterotypic and multitypic immunity are comprehensively identified, vaccine-induced protection should be demonstrated in pre-clinical studies, using animals with less genetic variability, studying the quality of the immune response, and using a rigorous and standardize dengue challenge.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fimmu.2014.00452/abstract

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Mouse models to study dengue virus immunology and pathogenesis

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The development of a compelling murine model of dengue virus (DENV) infection has been challenging, because DENV clinical isolates do not readily replicate or cause pathology in immunocompetent mice. However, research using immunocompromised mice and/or mouse-adapted viruses allows investigation of questions that may be impossible to address in human studies. In this review, we discuss the potential strengths and limitations of existing mouse models of dengue disease. Human studies are descriptive by nature; moreover, the strain, time, and sequence of infection are often unknown. In contrast, in mice, the conditions of infection are well defined and a large number of experimental parameters can be varied at will. Therefore, mouse models offer an opportunity to experimentally test hypotheses that are based on epidemiological observations. In particular, gain-of-function or loss-of-function models can be established to assess how different components of the immune system (either alone or in combination) contribute to protection or pathogenesis during secondary infections or after vaccination. In addition, mouse models have been used for pre-clinical testing of anti-viral drugs or for vaccine development studies. Conclusions based on mouse experiments must be extrapolated to DENV-infection in humans with caution due to the inherent limitations of animal models. However, research in mouse models is a useful complement to *in vitro* and epidemiological data, and may delineate new areas that deserve attention during future human studies.

Keywords: dengue, vaccines, mouse models, antibody-dependent enhancement, adaptive immunity

INTRODUCTION

Dengue virus (DENV) is the etiologic agent of the self-limited febrile illness dengue fever (DF), as well as the potentially lethal severe dengue disease (previously referred to as dengue hemorrhagic fever and dengue shock syndrome, DHF/DSS). Infection with DENV is often asymptomatic in humans (1). Symptomatic infections are characterized by: fever, retro-orbital headache, muscle, joint and bone pain, nausea, vomiting, abdominal pain, mucosal bleeding, and low platelet count. In the most severe form of the disease, severe bleeding, organ dysfunction, vascular permeability, and shock can occur (2, 3).

The four serotypes of DENV are the most prevalent mosquito-borne viruses that affect humans (4). In tropical and sub-tropical regions, it is estimated that 2.5–3.6 billion people are at risk of infection in over 100 countries (2, 5, 6). The development of an adequate mouse model for dengue infection has been challenging, mainly because DENV clinical isolates fail to replicate or cause pathology in wildtype (WT) mice. A reliable mouse model of DENV-infection would be an excellent complement to *in vitro* data and epidemiological studies, allowing for testing of hypotheses based on human observations, and providing insights into mechanisms of pathogenesis and immunity to DENV.

EXISTING ANIMAL MODELS OF DENV-INFECTION

Developing a relevant animal model for DENV-infection has been a long-standing challenge. The lack of an adequate animal model for DENV-infection is often mentioned as a major hurdle to a better understanding of DENV pathogenesis in humans. Numerous efforts to overcome this difficulty have resulted in many animal models. Though not perfect, these models have been useful to complement *in vitro* and human studies.

NON-HUMAN PRIMATE MODELS OF DENV-INFECTION

Non-human primate (NHP) models of DENV-infection exist, and have been recently reviewed (7). Viremia and humoral immune responses can be detected in NHP after DENV-infection, but usually no clinical signs of disease are observed (8–12). One study reported hemorrhage after infection with DENV (13). Due to the absence of signs of disease, it is difficult to study DENV-induced pathology in NHP, but NHP can be used for testing immunogenicity, safety, and/or protective efficacy of dengue vaccine candidates by measuring induction of anti-DENV antibodies and magnitude and duration of viremia after vaccination or challenge (14–23). Thus, in the context of vaccination, the antibody and viremia data from NHP models have been used to provide correlates of protection from infection but not from pathogenesis.

WILDTYPE MOUSE MODELS OF DENV-INFECTION

In many WT mouse models, intravenous (i.v.) or intraperitoneal (i.p.) DENV-infection results in neurological abnormalities but not the usual clinical signs observed in humans (24–27). Involvement of the central nervous system during DENV-infection in humans has generally been considered unusual (28, 29), although recent studies [reviewed in Ref. (3)] suggest that it is a frequent

complication (30–33). Central nervous system involvement is now considered criteria for severe dengue in the World Health Organization (WHO) case classification (2, 3). Intracranial (i.c.) inoculation of DENV in WT mice also results in neurological symptoms and death (34–37), however this inoculation route does not accurately mimic natural infection, which occurs when DENV-infected mosquitoes feed on a human. In C57BL/6 mice, a high inoculum of the non-mouse-adapted DENV2 strain 16681 resulted in systemic hemorrhage after intradermal (i.d.) infection (38) and abnormal liver function after i.v. infection (39). It is still a matter of debate which experimental route of infection (i.d. or i.v.) is the most relevant. While neither perfectly mimics the bite of an infected mosquito, i.d. and i.v. routes of infection are often used in laboratories when mosquito-mediated infection may not be possible. In another immunocompetent mouse model, presence of DENV was transiently detected in the serum of ICR, ddY, and Balb/c mice after i.p. injection of DENV-infected K562 cells (40). Collectively, the absence of overt signs of disease in WT mice after DENV-infection has been a major drawback of WT models to date.

MICE DEFICIENT IN IFN-α/β AND -γ RECEPTORS (AG129) TO STUDY DENV-INFECTION

In humans, DENV inhibits IFN signaling to establish infection, whereas DENV is unable to do so in mouse cells (41–43). Specifically, DENV NS5 can bind to and degrade human STAT2 (44), but not mouse STAT2 (41) and the DENV NS2B3 proteolytic complex can degrade human STING but not its mouse homologue (42, 43). The extreme sensitivity of DENV to type I IFN is highlighted by a recent study demonstrating effective viral clearance in mice deficient in both IRF-3 and IRF-7, which mount a weak and delayed type I IFN response during DENV-infection (45). Another study has demonstrated in mice that type II IFN efficiently limits DENV spread (46). Taken together, the high sensitivity of DENV to IFN and the fact that DENV inhibits IFN signaling in humans but not in mice could possibly explain why DENV clinical isolates do not easily replicate in WT mice.

In contrast to WT mice, mice deficient in IFN-α/β and -γ receptors on 129/Sv genetic background (AG129) support robust levels of DENV replication (47), and have thus widely been used as a mouse model of DENV-infection. Intraperitoneal infection with a mouse-adapted DENV-strain was lethal in AG129 mice, regardless of their age (47). While i.p. may not accurately mimic the natural route of infection, similar results were obtained in subsequent studies using i.v. inoculation. In those studies, i.v. infection of AG129 mice with a mouse-adapted DENV serotype 2 strain (DENV2) resulted in non-paralytic lethal dengue disease with signs of vascular permeability and TNF release (48, 49). This model has been used to demonstrate that passive transfer of sub-protective levels of anti-DENV antibodies before infection can turn a mild illness into a lethal disease recapitulating many signs of severe dengue disease, including viremia, cytokine storm, low platelet counts, elevated hematocrit, increased vascular permeability, and intestinal hemorrhage (50, 51). AG129 mice were also used to demonstrate the therapeutic efficacy of anti-DENV antibodies lacking the ability to bind the Fcγ receptor (52). Recently, i.p. infection with a non-mouse-adapted DENV2 strain (D2Y98P) was shown to cause cytokine storm, organ damage, and vascular leakage in AG129 mice (53, 54). If infected with lower doses of DENV2, AG129 mice do not develop severe dengue-like lethal disease, but instead neurological symptoms appear and result in paralysis later on (46, 51).

MICE DEFICIENT IN IFN-α/β RECEPTORS (IFNAR$^{-/-}$) TO STUDY DENV-INFECTION

In contrast to AG129 mice lacking both IFN-α/β and -γ receptors, mice lacking IFN-α/β receptor only (IFNAR$^{-/-}$) do not develop paralysis after i.v. infection with DENV2 (mouse-adapted) because IFN-γ and CD8$^+$ T cells prevent DENV-induced pathology in the central nervous system (46, 55). Instead of paralysis, these mice develop a severe dengue-like disease when infected with sufficiently high DENV2 challenge doses (46) or in the presence of sub-neutralizing (and enhancing) anti-DENV antibodies (51). IFNAR$^{-/-}$ mice have also been used to demonstrate that both CD4$^+$ and CD8$^+$ T cells play a protective role in the context of primary DENV2 infection (mouse-adapted strain, i.v. infection) and peptide vaccination (56, 57).

Recently, IFNAR$^{-/-}$ mice have been crossed with transgenic mice expressing human HLA molecules in order study anti-DENV-T cell responses restricted by human HLA (58). Observations made in those mice confirmed the role of NS3 and NS5 as major targets of the T cell response in humans (59), identified several novel human DENV-T cell epitopes, and highlighted the dominance of HLA-B*0702 restricted responses (58).

Sustained DENV replication and severe dengue disease manifestations in IFNAR$^{-/-}$ and AG129 mice are a clear advantage to study DENV pathogenesis *in vivo*, but the absence of intact IFN signaling is a limitation that must be taken into account when interpreting data. In particular, immune responses to DENV are altered in IFNAR$^{-/-}$ and AG129 mice compared to WT mice, as IFN is a key component of the immune system. Therefore, findings made in IFNAR$^{-/-}$ and AG129 mice may not accurately reflect what would happen in a fully immunocompetent environment. It would be desirable to validate the findings made in IFN-deficient mice with another model, or with data from epidemiological studies.

"HUMANIZED" MICE TO STUDY DENV-INFECTION

Another approach aimed at modeling DENV-infection in mice has been to graft human tumor cells into severe combined immunodeficient (SCID) mice lacking T and B cells. As SCID mice are unable to reject the graft, the transplanted human tumor cells provide a permissive environment for DENV replication. DENV replication was observed after transplantation of human hepatocarcinoma hepG2 (60) or human leukemia K562 cells (61). Viremia and some signs of disease (mild hemorrhage, thrombocytopenia, and elevated TNF levels) were detected in one study (60), but ultimately mice succumbed to paralysis. As DENV replication is restricted to the transplanted transformed human cells, extrapolation of results to human disease may be difficult.

Human CD34$^+$ hematopoietic stem cells have also been used to reconstitute irradiated NOD/SCID (62) or NOD/SCID/IL-2Rγ-null (63–65) mice. Graft-rejection is minimized in NOD/SCID/IL-2Rγ-null mice due to dramatic defects in both adaptive and innate immune systems. Therefore in irradiated NOD/SCID/IL-2Rγ-null mice, the efficiency of reconstitution by human CD34$^+$ cells is

maximized. In the resulting "humanized" chimeras (hu-NSG), human cells of the adaptive and innate immune systems develop with various degrees of reconstitution depending on the animal, the organ, and the laboratory (62, 66, 67). Fever, rash, viremia, erythema, and thrombocytopenia have been observed in humanized mice after DENV-infection (62, 63, 65), and human cells were infected in the bone marrow, spleen, and blood (62). Production of anti-DENV IgM and cytokines was also observed in those mice (64, 65, 68). Similar to NOD/SCID/IL-2Rγ-null mice, immunodeficient RAG2$^{-/-}$/γc$^{-/-}$ mice can also be reconstituted with human hematopoietic stem cells (67). DENV-infection of humanized RAG2$^{-/-}$/γc$^{-/-}$ resulted in viremia, fever, and production of human IgM and IgG (69). Recently, NOD/SCID/IL-2Rγ-null mice have been co-transplanted with cord blood hematopoietic stem cells and human fetal thymus and liver tissues, which resulted in higher immune responses to DENV-infection (neutralizing IgM and IFN-γ production) (70). In another study, NOD/SCID mice were grafted with human fetal liver and thymus prior to reconstitution with human CD34^{+} hematopoietic stem cells (71). In those mice, infection with DENV resulted in viremia, infection of human leukocytes, cytokine production, and production of DENV-specific IgM. Also, viral RNA levels were reduced by administration of an adenosine nucleoside inhibitor of DENV, either simultaneously or after infection (71). Based on those results, the authors suggest that "humanized" mice could be used to test the efficacy of anti-viral drugs or, potentially, the protective efficacy of vaccine candidates.

While using mice reconstituted with human cells offers the possibility to study DENV-infection of human cells *in vivo*, this approach is labor-intensive and important variations are observed in the degree of reconstitution of the recipients (62, 66, 67). In addition, cellular and molecular interactions between the grafted human cells and the murine environment may be absent or altered compared to the interactions that would take place in a natural environment. Therefore, transferred human cells may not function in the murine environment as they would in their natural (human) environment. Overall, few signs of severe dengue disease are observed in "humanized" mice, and some aspects of both adaptive and innate responses are not fully functional (67). Cellular immune responses are usually observed after viral infection, but isotype class-switch does not readily occur in these mice (67).

Various mouse models of DENV-infection exist, each one with specific strengths and limitations. While improvement of the current models would be desirable, these models have been used to test anti-viral drugs and vaccine candidates, or to provide insights into mechanisms of DENV pathogenesis. As pathogenesis and immune responses after DENV-infection probably differ between mice and humans, extrapolation of findings made in mice to DENV-infection in human should be done with caution. This limitation has to be kept in mind when reviewing any data generated in animal models.

USE OF MOUSE MODELS FOR DRUG AND VACCINE DEVELOPMENT

Efforts to develop efficient therapeutics and vaccines against DENV are still ongoing. Studies using mouse models of DENV-infection could be used for drug development, or provide valuable information on safety, immunogenicity, and efficacy of vaccine candidates before they reach the clinical testing stage.

ANTI-VIRAL TESTING IN MOUSE MODELS OF DENV-INFECTION

Several anti-viral drug candidates have been tested in WT mice after i.c. infection, or in AG129 mice. The alpha-glucosidase inhibitor castanospermine (a natural alkaloid) can inhibit DENV-infection by preventing correct folding of viral structural proteins (72) and prevent mortality after i.c. DENV challenge in WT mice (73). Contrary to WT mice, in which DENV is lethal only after i.c. challenge, AG129 mice support DENV replication and show signs of severe dengue disease following i.p. or i.v. infection with certain DENV-strains (48–54). Therefore, this model has been widely used to test the ability of anti-viral drugs to limit DENV replication and dissemination, and/or prevent signs of disease. Mortality, viral load, and signs of disease can all be used as readouts for the drug efficacy. Iminosugars have anti-viral properties, as they prevent correct protein folding by inhibiting glycoprotein processing enzymes α-glucosidases I and II (74). In AG129 mice, various iminosugars have been shown to reduce viremia, prevent cytokine storm, and/or limit mortality after DENV-infection (75–80). One study showed that co-administration of ribavirin enhanced the anti-viral activity of the iminosugar (76). Furthermore, an adenosine nucleoside could limit viremia and reduce mortality in DENV-infected AG129 mice, presumably by blocking viral RNA synthesis (81, 82). Another compound, which inhibited RNA translation, reduced peak viremia in AG129 mice (83). Other DENV inhibitors targeting the NS3 helicase (84) or the capsid protein (85) have also successfully reduced viremia and organ viral titers in AG129 mice. In recent years, AG129 mice have increasingly become the standard mouse model for *in vivo* testing of anti-viral candidates that impact viral replication through type I and II IFN-independent pathways.

MOUSE MODELS TO TEST SAFETY OF DENV-VACCINE CANDIDATES

A successful vaccine should induce protective immunity in the host without causing pathology. This is particularly true for live-attenuated vaccines, which are often derived from more virulent parental strains. Mouse models in which DENV can replicate and cause disease can be used to assess the safety of vaccine candidates. Duration and magnitude of viremia, morbidity, and (potentially) mortality after vaccination can be used as readouts to assess the degree of attenuation of vaccine candidates compared to the parental, non-attenuated strain. The degree of attenuation of live DENV-vaccine candidates has been tested in suckling mouse brains (86) and in SCID mice transplanted with human liver cells (18, 87, 88). The WHO has suggested that the AG129 mouse model may be adequate to test the safety of live-attenuated DENV-vaccine candidates, as the potential to cause disease (compared to the parental, non-attenuated strain) can be evaluated in those mice (89). However, the authors point out the difficulty of interpreting the results, as AG129 mice lack both type I and II IFN receptors. In addition, the risk of inducing antibody-dependent enhancement after vaccination can readily be assessed in AG129 mice, as viral titers and morbidity have been shown to increase when sub-neutralizing amounts of anti-DENV antibodies are administered prior to infection (50–52, 90).

MOUSE MODELS TO TEST IMMUNOGENICITY AND EFFICACY OF DENV-VACCINE CANDIDATES

Immunogenicity and efficacy of DENV-vaccine candidates have also been tested in both AG129 and WT mice. The effect of immunization on subsequent challenge can easily be observed in AG129 mice, as the appropriate DENV-strain will replicate to measurable levels and cause pathology in those mice. A protective vaccine should reduce viral titers and pathology, as well as increase mean survival time after lethal challenge. Live-attenuated monovalent and tetravalent vaccine candidates induced neutralizing antibodies in AG129 mice, and protected from lethal DENV challenge (23, 91, 92). One study reported induction of DENV-specific $CD8^+$ T cells after vaccination of $IFNAR^{-/-}$ mice (23). Recently, immunization with a non-propagating alphavirus replicon particle expressing a truncated version of the DENV envelope protein induced both cellular and humoral immunity, and was able to protect AG129 mice form lethal i.v. DENV challenge (90). The limitation of using AG129 mice is that vaccine-induced responses may be altered relative to immunocompetent mice or humans, as both type I and II IFN may regulate vaccine-induced immune responses.

Immune responses after vaccination with non-replicating DENV-vaccine candidates have also been investigated in WT mice, and neutralizing antibody responses were elicited after immunization with subunit vaccines containing portions of the DENV envelope protein (20, 93, 94) or after DNA priming and protein boosting with various combination of envelope and non-structural proteins (95, 96). Many studies used i.c. lethal DENV challenge to test the protective efficacy of DENV-vaccine candidates in WT mice (20, 34, 35, 37, 97–99). In those studies, the readout for protection was reduction of morbidity and/or increased survival after i.c. lethal challenge.

In general, testing the efficacy of DENV-vaccination in WT mice is difficult, as DENV challenge will not result in measurable viral titers or pathology in WT mice, with the exception of viral replication in the central nervous system after i.c. challenge (as mentioned above). One way to circumvent this problem is to use an adoptive transfer system: since many vaccines are immunogenic in WT mice, WT animals can be vaccinated and, subsequently, T cells, B cells, or serum can be transferred (alone or in combination) into naïve AG129 recipients prior to challenge. The protective or potentially enhancing effect of immune T cells, B cells, or serum can be assessed by monitoring changes in viral load or pathology in the AG129 recipient, where DENV readily replicates and causes disease. Thus, in this adoptive transfer system, the AG129 mice serve as a highly stringent challenge assay. This approach has been recently used to assess the relative contribution of T cells and antibodies after vaccination, revealing that not only antibodies, but also T cells could efficiently contribute to protection after vaccination (90).

MOUSE STUDIES TO COMPLEMENT HUMAN STUDIES

In vitro experiments cannot fully reflect the complexity of the whole organism, and human studies are descriptive by nature. In human studies, key parameters such as viral strain, dose, sequence of infection, and/or interval between infections are often unclear or cannot be easily manipulated. Therefore, research in mouse models is an ideal complement as it uses whole organisms, and allows for variation of many experimental parameters. Care must be taken while extrapolating conclusions based on experiments carried out in mice to dengue disease in humans, as there is some artificial component in all the models described so far.

MOUSE MODELS TO CONFIRM OBSERVATIONS MADE IN HUMAN STUDIES

Research in mouse models can be useful to verify hypotheses that have been based on epidemiological observations, or to gain insights into the mechanistic aspects of phenomena that have been observed in humans. For example, the hypothesis that antibody from a previous infection with a heterotypic virus (or acquired from an immune mother) can increase severity of disease upon re-infection (antibody-dependent enhancement, ADE) has been postulated a long time ago (100, 101). Increased infection of target cells in the presence of sub-neutralizing amounts of antibody is readily observed *in vitro* (102–106). In monkeys, viremia increases after transfer of antibodies, but no signs of disease are observed (107, 108). Recently, the AG129 mouse model was used to demonstrate that passive transfer of sub-neutralizing amounts of anti-DENV antibodies could turn a mild illness into a lethal disease upon DENV-infection (50, 51). The antibody-induced severe dengue disease displayed many signs observed during severe dengue disease in humans: elevated viral RNA titers in multiple organs, cytokine storm, low platelet counts, elevated hematocrit, increased vascular permeability, intestinal hemorrhage, and ultimately death. Those studies also formally confirmed the involvement of the Fcγ-receptor during ADE *in vivo*, and a subsequent study demonstrated the therapeutic potential of anti-DENV antibodies that no longer bind to the Fc-γ receptor (52).

Another example is the association between high viremia and disease severity. In humans, viremia is higher and persists longer during severe dengue compared to DF (109–113). This has also been observed in mice, where disease severity has been shown to correlate with higher viremia. Mouse models have been used to explore various experimental scenarios in which viral or host factors influence virus levels and, consequently, severity of disease. In the single-deficient $IFNAR^{-/-}$ mice, severity of disease correlated with the amount of virus inoculated i.v. (46). The same study showed that, at equal viral doses, the presence of intact IFN-γ signaling reduced systemic viral spread and severity of disease. Other mouse model studies examining mechanisms of anti-viral innate immunity revealed an essential role for MAVS (55) and IRF-3/7 (45) in the initial induction of type I IFN response and control of viral replication, and for STAT1 and STAT2 in the late induction of type I IFN response and control of viral replication (114). In various studies using IFN-α/β and -γ receptor deficient mice, the presence of sub-neutralizing levels of antibodies increased viremia and resulted in severe dengue-like disease (50–52). CD8-depletion before infection resulted in increased viral loads in another murine study (56). Many studies have also shown that virulence factors influence the outcome of the infection. One study showed that two mutations in the envelope protein of DENV were sufficient to delay virus clearance from serum, increase systemic viral loads, and induce severe dengue disease in mice (49). Similarly, a single amino acid change in the non-structural protein NS4B of a non-virulent DENV-strain caused increased RNA synthesis, increased viremia,

and decreased survival time in AG129 mice (54). Therefore, while the observation that higher viremia correlates with disease severity has been widely made in humans, studies in mice have confirmed and extended those observations by demonstrating how particular host- and virus-associated factors impact viremia and disease severity; those factors include specific elements of innate and adaptive immune responses, presence of sub-neutralizing antibodies, size of the initial virus inoculum, and virulence of the infecting viral strain.

MOUSE MODELS TO EXPLORE THE ROLE OF DIFFERENT COMPONENTS OF THE IMMUNE SYSTEM

Human studies have suggested that the immune system can be involved in both protection and immunopathology during DENV-infection. For example, studies have shown that passively transferred anti-DENV maternal antibodies can initially protect infants against disease (115–118), but later on, as titers decrease, sub-protective levels of antibody favor the development of severe disease (115, 116, 118). T cells have also been suspected to increase disease severity: cross-reactive T cells raised during a primary infection have been suggested to dominate the response during a secondary, heterotypic infection, resulting in delayed viral clearance (119), altered cytokine production profile (120), or excessive cytokine release (121). However, another study showed that while cross-reactive T cells did dominate the response during secondary heterotypic infections, they did not show any sign of impairment (122). On the contrary, results of this study supported an HLA-linked protective role for $CD8^+$ T cells.

Based on these observations, it is clear that a better understanding of the relative contribution of the cellular and the humoral arms of the immune system to protection and/or immunopathogenesis during DENV-infection is needed. Tractable, genetically manipulable mouse models enable the investigation of the role of different components of the immune system during DENV-infection, re-infection, or after vaccination. Passive transfer of serum, adoptive transfer of various T cell subsets, or depletion experiments should allow for a better understanding of the protective or potentially pathogenic role of the different components of the immune system, either alone or in combination. Passive transfer of anti-DENV antibodies can protect mice from intracerebral lethal challenge (123) or increase mean survival time after i.v. challenge (51). However, sub-neutralizing levels of antibodies can also increase the level of infection and/or the severity of disease in mice (50, 51). One study showed that the same neutralizing antibody could protect or enhance infection, depending on the amount transferred prior to infection (51). This is an *in vivo* confirmation of the observation made *in vitro* that even antibodies that are neutralizing can enhance infection when used at sub-neutralizing concentrations (103). A protective role for T cells during DENV primary infection was established in mice by showing that CD8-depletion prior to infection increased viral load in various organs upon infection (56). Similarly, vaccination with CD8-epitopes (56) or CD4-epitopes (57) reduced viral load upon DENV-infection, showing that priming cellular responses before challenge was beneficial for the host. Passive transfer of homotypic or heterotypic serum or adoptive transfer of homotypic or heterotypic splenocytes before DENV challenge reduced viral load in various organs (124). Similarly, a protective role for both cross-reactive T cells and B cells was shown in AG129 mice (125). Recently, the relative contribution of the humoral and the cellular arms of the immune system were assessed in mice after vaccination with a protective DENV-vaccine candidate (90). After vaccination, short-term protection was mediated by $CD8^+$ T cells. Later on, $CD8^+$ T cells were or were not required for protection, depending on the immunization schedule. All those studies explored the role of various components of the immune system in isolation from the other ones, which would be difficult in human studies.

Despite sustained vaccine development efforts, there is still no clinically approved vaccine against DENV (126–133). In terms of dengue vaccine development efforts to date, measuring neutralizing antibodies by plaque reduction neutralization test (PRNT) may not accurately predict the protective efficacy of a vaccine against DENV (133–135). Therefore, a better understanding of the relative contribution of the different components of the immune system to protection and/or pathogenesis is crucial to develop better correlates of protection as well as a safe and effective DENV vaccine.

MOUSE MODELS TO SUGGEST AREAS OF INTEREST FOR FUTURE HUMAN STUDIES

While research in mouse models has confirmed some of the hypotheses based on human studies, some of the findings made using mouse models need validation with human data. In this respect, the value of the mouse model is to point out possible areas of importance for future human studies. For example, elevated liver DENV titers and infection of liver sinusoidal endothelial cells (LSECs) were observed during antibody-induced severe dengue disease in mice (51). In humans, liver pathology is often observed after DENV-infection, and the degree of dysfunction may correlate with disease severity (136–140). In addition, DENV RNA or antigen has been found in the liver of patients who succumbed to DHF/DSS (141–143). Recently, a case of DENV transmission following a liver transplant has been reported (144). However, the cellular localization of DENV replication in the liver is still controversial, and possibly deserves more attention in future human studies.

Similarly, a protective role for T cells during DENV-infection has been widely shown in mice (56, 57, 90), but human studies have only recently started to explore more in depth the protective role of T cells during DENV-infection (122). Studies in mice could suggest which T cell subsets are required to limit viral replication, and which T cell-mediated mechanisms protect against dengue pathogenesis during both primary and secondary infections, as well as explore further the role of sero-specific and cross-reactive T cells in protection and/or pathogenesis. $IFNAR^{-/-}$ mice crossed to transgenic mice expressing human HLA molecules will also be valuable to explore anti-DENV-T cell responses restricted to human HLA. Subsequently, human studies will be necessary to verify if the findings made in mice apply to DENV-infection in humans.

CONCLUSION

While the perfect mouse model of DENV-infection has yet to be established, existing murine models of DENV-infection have, within their own limitations, been invaluable tools to complement

and/or expand observations made *in vitro* or in epidemiological studies. Each model has strengths and weaknesses that must be taken into consideration when assessing data. Therefore, the choice of the best-suited model to answer a particular question is of critical importance. Mouse models have been useful to test the safety and efficacy of vaccine candidates or potential anti-viral drugs, verify hypotheses based on human studies, gain insights into mechanistic aspects of DENV-induced immunity or pathology, and suggest areas worth attention in future human studies. As with any animal model, extrapolation of findings made in mice to DENV-infection in humans must be done with care. In the future, refinement and improvement of existing models may overcome some of the boundaries of today's models, and expand the knowledge that murine models can generate about mechanisms of DENV pathogenesis and immunity.

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A rapid immunization strategy with a live-attenuated tetravalent dengue vaccine elicits protective neutralizing antibody responses in non-human primates

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Dengue viruses (DENVs) cause approximately 390 million cases of DENV infections annually and over 3 billion people worldwide are at risk of infection. No dengue vaccine is currently available nor is there an antiviral therapy for DENV infections. We have developed a tetravalent live-attenuated DENV vaccine tetravalent dengue vaccine (TDV) that consists of a molecularly characterized attenuated DENV-2 strain (TDV-2) and three chimeric viruses containing the pre-membrane and envelope genes of DENV-1, -3, and -4 expressed in the context of the TDV-2 genome. To impact dengue vaccine delivery in endemic areas and immunize travelers, a simple and rapid immunization strategy (RIS) is preferred. We investigated RIS consisting of two full vaccine doses being administered subcutaneously or intradermally on the initial vaccination visit (day 0) at two different anatomical locations with a needle-free disposable syringe jet injection delivery devices (PharmaJet) in non-human primates. This vaccination strategy resulted in efficient priming and induction of neutralizing antibody responses to all four DENV serotypes comparable to those elicited by the traditional prime and boost (2 months later) vaccination schedule. In addition, the vaccine induced $CD4^+$ and $CD8^+$ T cells producing IFN-γ, IL-2, and TNF-α, and targeting the DENV-2 NS1, NS3, and NS5 proteins. Moreover, vaccine-specific T cells were cross-reactive with the non-structural NS3 and NS5 proteins of DENV-4. When animals were challenged with DENV-2 they were protected with no detectable viremia, and exhibited sterilizing immunity (no increase of neutralizing titers post-challenge). RIS could decrease vaccination visits and provide quick immune response to all four DENV serotypes. This strategy could increase vaccination compliance and would be especially advantageous for travelers into endemic areas.

Keywords: dengue, vaccine, non-human primates, neutralizing antibodies, needle-free delivery, T cell responses

INTRODUCTION

Millions of people living in tropical and subtropical parts of the world are infected with dengue viruses (DENVs) each year (1, 2). The dramatic spread of the disease has been mainly attributed to the geographical expansion of the mosquito vector combined with inadequate measures of vector control, increased human travel, and urbanization (3). DENVs circulate in nature as four distinct serotypes (DENV-1 to DENV-4), each capable of causing a spectrum of disease ranging from subclinical infection to dengue fever (DF), and sometimes to life-threatening dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS) (1, 4, 5). Normally, infection with one dengue serotype will confer long-term protection against reinfection by the same serotype. However, in case of reinfection by a heterologous dengue serotype, there is the potential risk of antibody-dependent enhancement (ADE) of disease associated with the presence of cross-reactive antibodies (6) and/or cross-reactive T cells (7, 8). Therefore, vaccine development against DENV has focused on tetravalent formulations that can simultaneously provide protection to all four DENV serotypes.

Currently, there are several candidate DENV vaccines at various stages of preclinical and clinical testing (9). This article describes a live-attenuated tetravalent dengue vaccine (TDV) consisting of a molecularly characterized attenuated DENV-2 strain (TDV-2) and three chimeric viruses containing the pre-membrane and envelope genes of DENV-1, -3, and -4 expressed in the context of the TDV-2 genome (TDV-1, TDV-3, and TDV-4, respectively) (10–15). TDV has been extensively tested in preclinical studies (16, 17), two completed Phase 1 clinical trials, and is currently tested in Phase 2 clinical trials in dengue endemic areas. It was shown to be well-tolerated in healthy adults and induced neutralizing

antibody responses to all four dengue serotypes (Osorio et al., in preparation; George et al., in preparation).

To improve dengue vaccine delivery globally and in diverse clinical settings an easy delivery method is required combined with a vaccination schedule that will improve compliance. Delivery approaches such as those using jet injectors have been considered as alternatives to the conventional needle and syringe (N–S) injection, with some on the market, and others being tested in clinical trials (18). In this non-human primates (NHP) study, we evaluated the administration of TDV via the subcutaneous (SC) or intradermal (ID) routes using a needle-free delivery device developed by PharmaJet (PhJ). In addition, we investigated rapid immunization strategy (RIS) to administer animals with double doses of vaccine (two separate injection sites, one dose at each site) on day 0 (0, 0). Our results indicated that this RIS strategy induced immune responses comparable to those elicited when two doses are given 53 days apart.

MATERIALS AND METHODS

VIRUSES AND VACCINES

DENV-2 New Guinea C (NGC) used as challenge virus in this study was generously provided by Dr. Steven Whitehead (National Institutes of Health, Bethesda, MD, USA). For neutralizing antibody assays, we used virus strains from which the prM and E genes of each live-attenuated dengue vaccine serotype were derived (DENV-1 16007, DENV-2 16681, DENV-3 16562, and DENV-4 1036). DENVs were grown in Vero cells or C6/36 cells in Dulbecco's modified minimal essential medium (DMEM) containing 5% fetal bovine serum (FBS) and penicillin–streptomycin.

The four vaccine viruses were generated from cDNA clone-derived DENV-2 VV45R virus (based on the genome of DENV-2 PDK-53), and the DENV-2 PDK-53-based chimeras expressing the prM and E genes of DENV-1 16007, DENV-3 16562, or DENV-4 1036. The construction and characterization of these viruses has been previously reported (10, 19).

NON-HUMAN PRIMATES

Twelve adult male, DENV seronegative cynomolgus macaques originating from Vietnam were used. The animals were placed in quarantine for 30 days prior to study start. The study was conducted at the Charmany Instructional Facility of the University of Wisconsin, Madison, WI, USA in compliance with the Animal Care Regulations.

EXPERIMENTAL ANIMAL STUDY DESIGN

In this study, groups of monkeys ($n = 3$) received the TDV formulated into either 0.5 ml for SC administration or 0.1 ml for ID delivery using the PhJ device. Each full dose of the tetravalent vaccine used in this study contained 2×10^4 PFU of TDV-1, 5×10^4 PFU of TDV-2, 1×10^5 PFU of TDV-3, and 3×10^5 PFU of TDV-4 vaccine viruses. This vaccine constitutes the clinical trial material used for two Phase 1 studies conducted in USA and Colombia, as well as Phase 2 studies currently ongoing in endemic areas.

Each animal in the first two groups received two injections on day 0, one in each arm ID or SC. A third group of animals was injected SC on day 0 and 53 using the PhJ device. Control animals received PBS via the ID route using PhJ. On day 90, all animals were challenged SC with 10^5 PFU of DENV-2 (NGC strain) using N–S. Serum samples were collected on days 0, 3, 5, 7, 10, 12, 14, 53, 64, 67, and 88 post-primary immunization to analyze vaccine viremia, and days 91, 93, 95, 97, 99, 101, 102, and 104 to analyze DENV-2 NGC viremia after challenge. Serum samples also were collected on days 0, 30, 53, 75, 88, and 104 to determine neutralizing antibody titers to each serotype. PBMCs from group 2 and 4 were collected to measure T cell responses.

SERUM VIRAL RNA

Viral RNA in serum samples was measured using a quantitative reverse transcription-polymerase chain reaction (qRT-PCR) as follows. Viral RNA was extracted from 140 μl of each individual serum sample using a QIAamp viral RNA kit (Qiagen, Valencia, CA, USA) and eluted in 60 μl elution buffer. Viral RNA standards, used to create a standard curve in all qRT-PCR assays, were *in vitro* transcribed from cDNA clones and quantified as previously described (20). E-gene primers, TaqMan probes, and RNA standards were serotype specific (**Table 1**). Using a different fluorophore for each serotype specific probe (sequences available upon request), qRT-PCRs were performed in duplex: one reaction quantified TDV-1 and TDV-2 vaccine viruses while a separate one quantified TDV-3 and TDV-4 viruses RNA. Following DENV-2 NGC challenge, viral RNA was quantified in a singleplex qRT-PCR. All qRT-PCR reactions were performed in a final volume of 25 μl using the QuantiTect Virus +ROX Vial Kit (Qiagen, Valencia, CA, USA). The reactions contained 5 μl extracted RNA, 0.4 μM of each primer, and 0.2 μM probe. The reaction was conducted in the iQ5 iCycler system (Bio-Rad Laboratories) using the following cycle; 1 cycle of 50°C for 20 min at room temperature (RT), 1 cycle of 95°C for 5 min, and 50 cycles of 95°C for 15 s. Limit of detection for the qRT-PCR was determined for each viral RNA standard by creating a standard curve consisting of nine replicates per dilution. While the sensitivity reached 3.9 copies/reaction (~2.7 $\log_{10}$ copies/ml), 3.6 $\log_{10}$ copies/ml met the criteria of a 100% detection

Table 1 | E protein primers used in this study.

	Sequence
ANTI-SENSE PRIMERS	
CD1-1593	CAA GGC AGT GGT AAG TCT AGA AAC C
CD2-2116	TCT TAA ACC AGT TGA GCT TCA GTT GT
CD3-2000	CCA CTG GAT TGG CTG TGA TC
CD4-843	GCG CGA ATC CTG GGT TT
SENSE PRIMERS	
D1-1459	GACCGACTACGGAACCCTTACAT
D2-1929	TCC ATG CAA GAT CCC TTT TGA
D3-1872	CGC AGC ATG GGA CAA TAC TC
D4-637	GCTGGTGCAATCTCACGTCTA
PROBES	
CD1-1519P	CTC GTT AAA ATC TAG CCC TGT CCT AGG TGA ACA AT – FAM
D2-2000P	ACC CAA TTG TGA CAG AAA AAG ATA GCC CAG TC – TET
D3-1914P	AAG ATG CAC CCT GCA AGA TTC CTT TCT C – TET
CD4-699P	TCC GTT CTC CGC TCT GGG TGC AT – FAM

rate as well as a low ($\leq$0.5) cycle threshold standard deviation of the replicates and was used as a cutoff for the assay.

MICRONEUTRALIZATION ASSAY

Serum samples were incubated at 56°C for 30 min to inactivate complement and possible adventitious agents. Heat-inactivated serum samples then were tested for neutralizing activity using a viral immunofocus reduction microneutralization assay and analyzed by an AID ELISpot reader (San Diego, CA, USA). Briefly, 96-well tissue culture plates were seeded with Vero cells at a density of 1.3×10^5 cells/ml in 100 µl/well. Cells were grown at 37°C in a 5% CO_2 incubator for 48 h. Twofold serial serum dilutions were prepared in a separate 96-well plate and then mixed with virus suspension containing 100 PFU followed by incubation at 4°C for 13–15 h. Culture medium was discarded from the Vero cell plates and then 30 µl of the serum–virus mixture was added to each well in triplicate followed by incubation at 37°C for 2 h. Control positive and negative serum samples were also included. An overlay medium with 1.2% carboxymethyl cellulose was added (100 µl/well) and cells were incubated as above for 2 days for DENV-4, 2.5 days for DENV-1 and -3, and 3 days for DENV-2. After incubation, the overlay was removed and cells were fixed with 85% cold acetone for 10 min at RT. Acetone was then discarded and plates were stored at −20°C until further use. Prior to staining, plates were equilibrated to RT, and washed three times with PBS to rehydrate the cells and to remove any residual overlay. Rabbit anti-DENV polyclonal antibody diluted (1:1000) in PBS-T containing 2.5% (w/v) dry milk powder was added, and plates were incubated at 37°C for 2 h. Plates were washed three times with PBS-T and incubated with anti-rabbit antibody conjugated with horse radish peroxidase (HRP) at 37°C for 2 h. Finally, plates were washed three times with PBS-T and incubated with the substrate (3-amino-9-ethylcarbozole) for 10–30 min or until plaques were visible. The plates were then washed with water and air-dried. The viral immunofoci were quantified on an ELISpot reader. Fifty percent of the average number of foci in the negative control serum defined the cutoff point. The serum dilution closest to the cutoff was recorded as the reciprocal neutralizing titer.

INTRACELLULAR CYTOKINE SECRETION ASSAY BY FLOW CYTOMETRY

To assess the functional capability of TDV-elicited dengue-specific T cells, we performed intracellular cytokine staining (ICS) assays. For positive control, we used Staphylococcus Enterotoxin B (SEB) stimulation, for negative control, we used tissue culture medium devoid of added stimulatory peptides. Peptide arrays used in this study (**Table 2**) were obtained from the National Institute of Allergy and Infectious Diseases Biodefense and Emerging Infections Research Resources Repository (BEI Resources). Individual peptides were prepared as 10 mM stock solutions for NS1 and NS5 and 15 mM for NS5 peptides. An aliquot of $0.5–1.5 \times 10^6$ PBMC in 200 µl total volume was incubated with peptides at 5 µM final concentration in the presence of anti-CD28 (clone L293), anti-CD49d (clone 9F10), and CD107a PE (clone H4A3) antibodies, and 1 µg per test of Brefeldin A (Sigma-Aldrich, St. Louis, MO, USA) and Golgi Stop at 37°C in a 5% CO_2 incubator overnight. Cells were stained for the surface expression of CD3 (PE-CF594 clone SP34-2), CD4 (PerCP-Cy5.5-conjugated clone L200), CD8 (Pacific Blue-conjugated clone RPA-T8), and live/dead fixable Aqua Dead Cell stain (Invitrogen), washed twice with FACS buffer, and fixed with 2% paraformaldehyde. Cells were then permeabilized with 0.1% saponin buffer, intracellularly stained for IFN-γ (Alexa Fluor 700-conjugated clone 4S.B3), TNF-α (FITC-conjugated clone Mab11), and IL-2 (APC-conjugated clone MQ1-17H12), washed twice with saponin buffer, and fixed with 2% paraformaldehyde. All fluorescent-labeled antibodies and reagents were purchased from BD Biosciences except when mentioned. Sample data were acquired on a SORP BD LSR II equipped with a 50 mW 405 violet, a 100 mW 488 blue, and a 50 mW 640 red laser (BD Biosciences) using FACSDiva version 6.1 acquisition software. We collected approximately 150–300 thousand events in the lymphocyte gate defined by forward and side scatter parameters. Data were analyzed by FlowJo™ 9.4.2 software (Tree Star, Inc., Ashland, OR, USA). Background values from peptide stimulated values were subtracted. The frequency of cytokine-positive T cells was presented as the percentage of gated $CD4^+$ or $CD8^+$ T cells.

Table 2 | Peptide arrays.

Serotype/peptides	aa. Number	Virus strain	Cat. no. (NBI)
DENV-2 NS1	47	New Guinea C	NR-508
DENV-2 NS3	83	New Guinea C	NR-509
DENV-2 NS5	155	New Guinea C	NR-2746
DENV-4 NS3	106	Singapore/8976/1995	NR-2756
DENV-4 NS5	156	Singapore/8976/1995	NR-4205

RESULTS

VACCINE VIRAL RNA FOLLOWING IMMUNIZATION

Following immunization, the presence of vaccine viral RNA in the serum was monitored by qRT-PCR of sequential bleeds collected over a period of 14 days post-primary immunization. TDV induced detectable TDV-2 virus replication from day 5 to 14 for animals injected SC, and day 7–12 for those injected ID (**Table 3**). No viral RNA from TDV-1, -3, and -4 vaccine viruses was detected in any of the groups on samples collected over a period of 14 days post-primary immunization.

NEUTRALIZING ANTIBODY RESPONSES ELICITED BY VACCINATION

The individual neutralizing antibody titers and kinetics of antibody responses elicited by TDV are shown in **Table 4**. Overall, administration of the vaccine by the ID or SC routes using the RIS (0, 0 vaccination schedule) induced comparable neutralizing antibody titers to all four serotypes. In all cases, the dominant neutralizing antibody response was to DEN-2, whereas TDV-4 was the least immunogenic component of the tetravalent vaccine formulation.

CHARACTERIZATION OF T CELL RESPONSES ELICITED BY THE VACCINE

To determine the target proteins of the T cell response elicited by TDV, PBMCs from immunized animals (group 2) collected on day 53 post-priming were restimulated *in vitro* with pools of peptides encompassing the entire sequence of DENV-2 NS1, NS3, and NS5 proteins (**Table 2**). As shown in **Figure 1**, $CD4^+$ T cells predominantly targeted the NS1 protein and to a lesser extent the NS3

and NS5 proteins, producing IFN-γ (a), IL-2 (b), and TNF-α (c). The vaccine also elicited $CD8^+$ T cells mainly recognizing epitopes from the NS1 protein and to a lesser degree from NS3 and NS5 proteins (**Figure 2**). In particular, responses to the NS1 were characterized by the production of IFN-γ (a), IL-2 (b), TNF-α (c), and expression of $CD107a^+$ marker (d). In contrast, T cell responses in PBS immunized animals (group 4) were comparatively very low (**Figures 1** and **2**). In addition, vaccine-specific $CD8^+$ IFN-γ producing T cells were cross-reactive with epitopes from the NS3 and NS5 non-structural proteins of DENV-4 (**Figure 3A**) and were shown to express the $CD107a^+$ marker (**Figure 3B**). A similar pattern of T cell responses recognizing predominantly the NS1 protein with no significant differences in frequencies of $CD4^+$ and $CD8^+$ T cells were also measured in group 3 (data not shown).

Table 3 | TDV-2 virus RNA detected in the serum after primary immunization with TDV.

Group	Dosing schedule	No. of animals positive for viral RNA				
		Day 5	Day 7	Day 10	Day 12	Day 14
1	TDV PhJ/ID (day 0, 0)	–	1/3 (4.8)	3/3 (4.5–4.9)	2/3 (3.9–4.3)	–
2	TDV PhJ/SC (day 0, 0)	1/3 (3.8)[a]	3/3 (4.0–4.3)	3/3 (3.7–4.7)	1/3 (4.0)	–
3	TDV PhJ/SC (day 0, 60)	1/3 (3.8)	3/3 (4.5–5.4)	3/3 (3.8–5.3)	3/3 (3.2–4.8)	2/3 (3.7–5.0)
4	PBS PhJ/ID (day 0, 60)	–	–	–	–	–

Results are averages from duplicate or triplicate data.

Samples with titers <3.6 log_{10} copies/ml were considered negative.

[a]*Data in parenthesis represent range of titers in log_{10} copies/ml.*

PROTECTION FROM DENV-2 NGC CHALLENGE

Since TDV-2 constitutes the backbone of TDV in this study, we examined the protective efficacy of this vaccine against challenge with DENV-2 NGC strain. Upon DENV-2 NGC challenge viral RNA was detected in the serum of all mock-immunized animals (**Table 4**). None of the vaccinated animals displayed DENV-2 NGC RNA. When the neutralizing antibody responses to DENV-2 were compared before and after challenge there was no significant increase in antibody titers in all vaccinated groups suggesting that DENVax elicits sterilizing immunity to DENV-2 (**Table 5**). In contrast, mock vaccinated animals developed a strong anti-DEN-2 neutralizing antibody response after challenge (**Table 5**).

DISCUSSION

To facilitate global dengue prevention and control through effective vaccination, a simple and practical method of administration is highly desirable. This study examined several aspects of vaccine delivery in the NHP model. In particular, we compared immune responses elicited by the SC and ID routes using a needle-free disposable syringe jet injection (DSJI) delivery device and assessed RIS as an alternative to the traditional prime/boost vaccination schedule. Immunization with TDV resulted in the detection of only TDV-2 virus RNA in the serum of vaccinated animals. This is consistent with our previous observations in the NHP model (17). The absence of post-boost viremia in animals that received a prime and booster immunization (0, 60) suggests that priming with the tetravalent vaccine was effective in eliciting immune responses able to reduce and control viral replication upon secondary exposure.

The use of a needle-free DSJI delivery device to administer TDV provided strong evidences suggesting the feasibility of an alternate approach to ID administration. Indeed, measurement of neutralizing antibody responses demonstrated that the vaccine was highly immunogenic. However, responses were unbalanced with anti-DEN-2 neutralizing titers being the highest and those against DEN-4 the lowest. This finding is consistent with previous

Table 4 | Kinetics of neutralizing antibody responses in animals vaccinated with TDV SC or ID using the PharmaJet device.

NHP ID	Vaccine regimen	Day 30				Day 53				Day 88			
		Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4
CY0503	0, 0 PhJ/ID	40	1280	160	20	160	1280	40	10	80	5120	20	40
CY0504		80	640	40	10	40	2560	40	5	80	2560	20	40
CY0505		320	1280	2560	320	160	640	640	160	80	640	160	40
	GMT	101	1016	254	40	101	1280	101	20	80	2032	40	40
CY0473	0, 0 PhJ/SC	2560	10,240	160	40	640	5120	80	20	320	1280	40	20
CY0474		1280	320	1280	160	640	640	640	160	320	320	160	80
CY0475		640	320	640	320	640	320	320	80	160	320	160	160
	GMT	1280	1016	508	127	640	1016	254	64	254	508	101	64
CY0493	0, 60 PhJ/SC	160	2560	160	20	80	2560	10	20	320	2560	80	40
CY0494		320	640	640	160	320	640	320	10	80	640	160	80
CY0495		640	2560	320	40	640	2560	40	80	1280	2560	160	40
	GMT	320	1613	320	50	254	1613	50	25	320	1613	127	50

GMT, geometric mean titer.

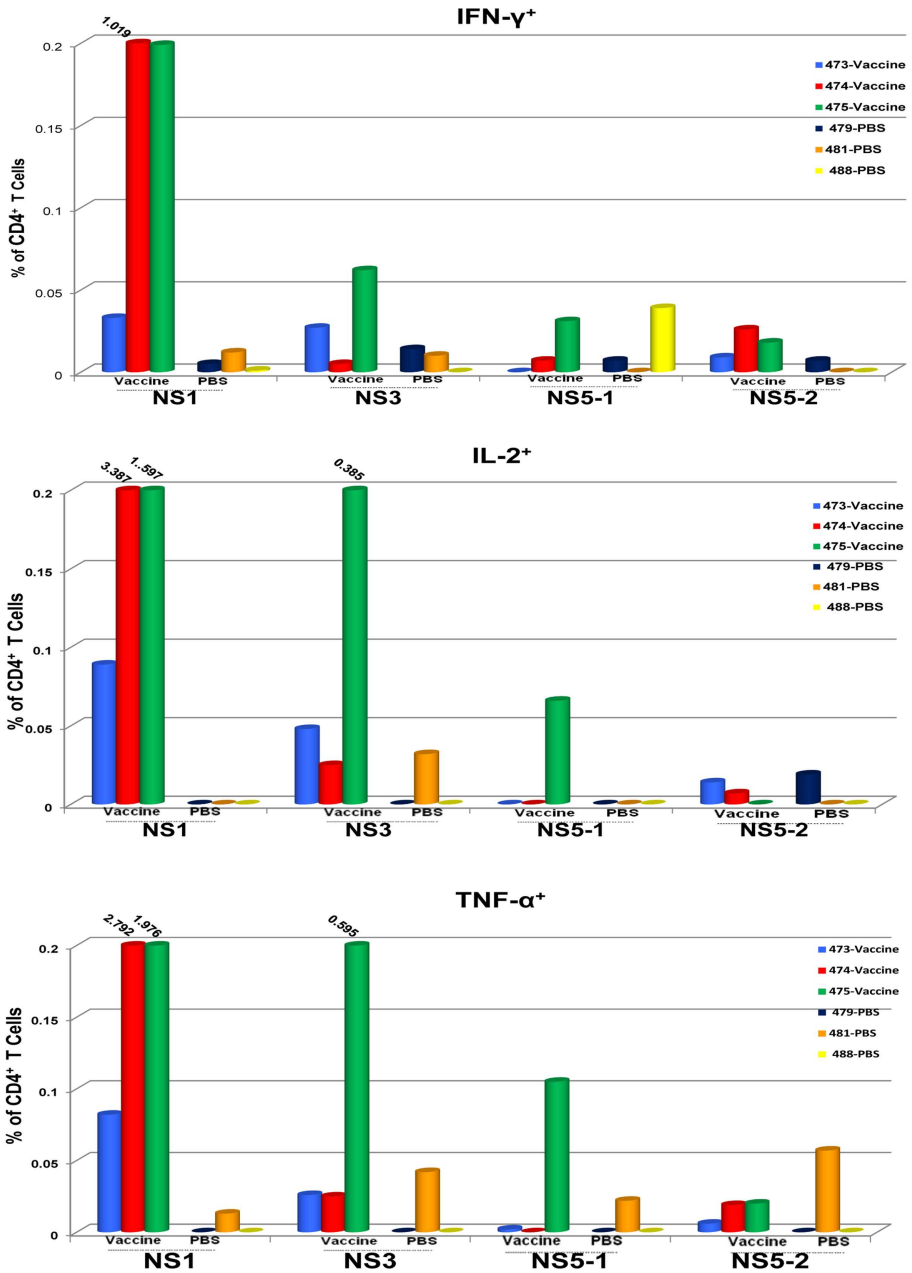

FIGURE 1 | CD4+ T cell responses to TDV target the non-structural proteins of TDV-2. Responses are shown as percentage of cytokine-positive T cells from DENV-2 peptide arrays stimulated PBMCs with the background percentage of cytokine-positive T cells in medium only treated cells subtracted. Peptide arrays for NS5 were split into two pools; NS5-1 and NS5-2. PBMCs from PBS immunized animals were used as controls.

observations made in preclinical animal models (10, 16, 17) and in Phase 1 clinical trials conducted in the USA and Colombia (Osorio et al., and George et al., in preparation) using N–S delivery with a prime/boost schedule. Since DENV-2 is the most frequent serotype implicated in DHF/DSS in secondary DENV infections (21) it could be argued that immunization with TDV could be advantageous in conferring protection against this serotype. When antibodies induced by the 0, 0 and 0, 53 vaccination schedule by the SC route were compared, the overall titers to all four DENV serotypes were similar. This suggests that the 0, 0 immunization schedule can efficiently prime the immune system for tetravalent responses, which can be sustained at high levels up to 3 months. Therefore, this vaccination schedule could be especially advantageous for travelers in endemic areas.

In the context of vaccination, it is critical to characterize the profile of T cell responses and determine the target proteins of this response. The recent analysis of T cell responses from a large cohort of DENV-infected individuals has highlighted the role of T

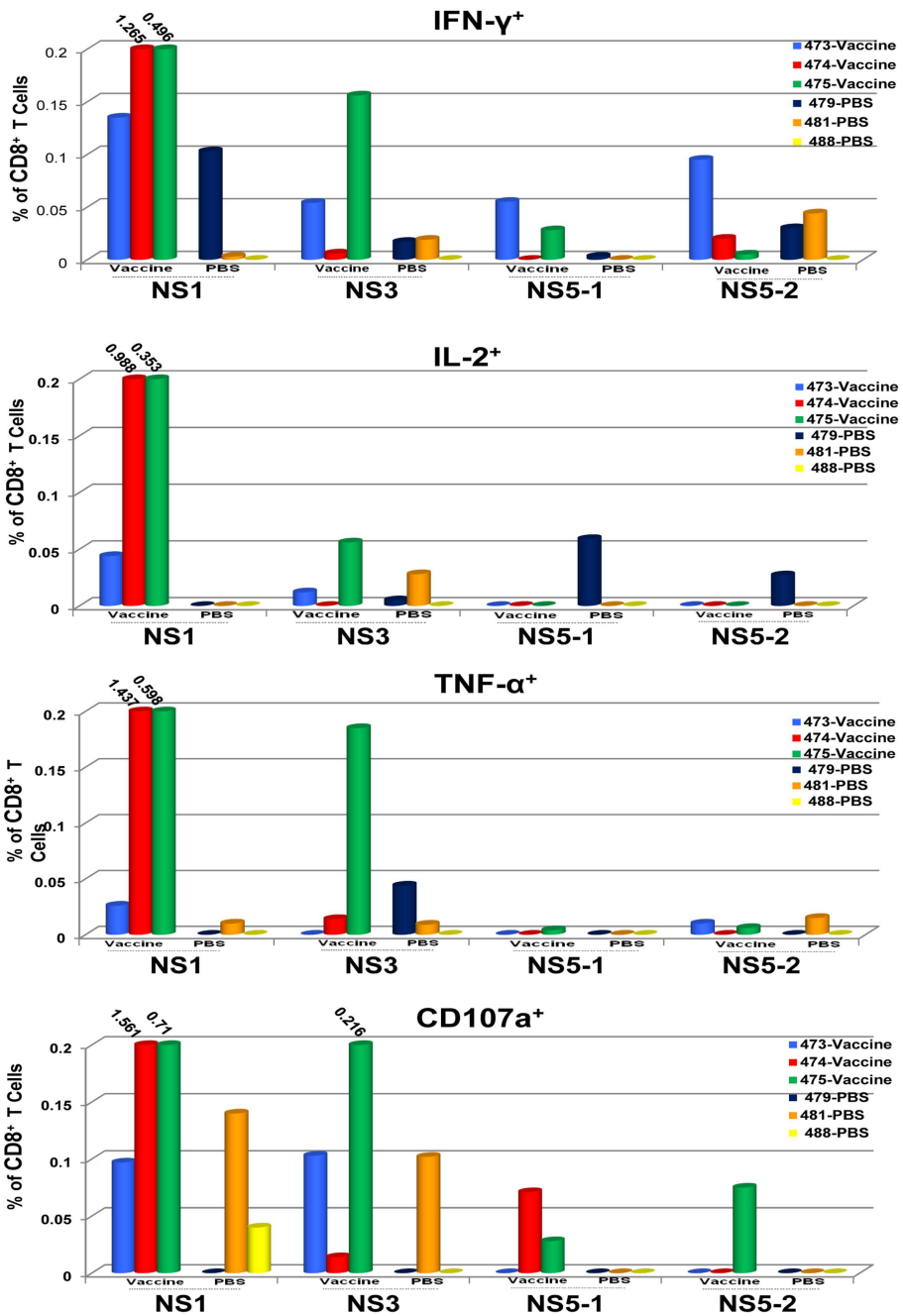

FIGURE 2 | CD8+ T cell responses to TDV target the non-structural proteins of TDV-2. Responses are shown as percentage of cytokine-positive T cells from DENV-2 peptide arrays stimulated PBMCs with the background percentage of cytokine-positive T cells in medium only treated cells subtracted. Peptide arrays for NS5 were split into two pools; NS5-1 and NS5-2. PBMCs from PBS immunized animals were used as controls.

cells in prevention of development of disease (22). In this study, we demonstrated that SC PhJ delivery of TDV using RIS is effective in inducing $CD4^+$ and $CD8^+$ T cells. Both T cell subsets produced IFN-γ, TNF-α, and IL-2 highlighting their Th1-type immune profile. In addition, using peptide arrays we demonstrated that they predominantly recognized sequences from the NS1 protein and to a lesser extent from NS3 of DENV-2. Moreover, we observed that the TDV-2 backbone elicited cross-reactive T cell responses to the highly conserved NS proteins of DENV-4. Similarly, we have observed cross-reactivity with the NS proteins of DENV-1 and E proteins of each serotype (data not shown). Overall, these findings highlight the potential of TDV-2 backbone to elicit a broad range of cross-reactive T cell responses to all four DENV serotypes.

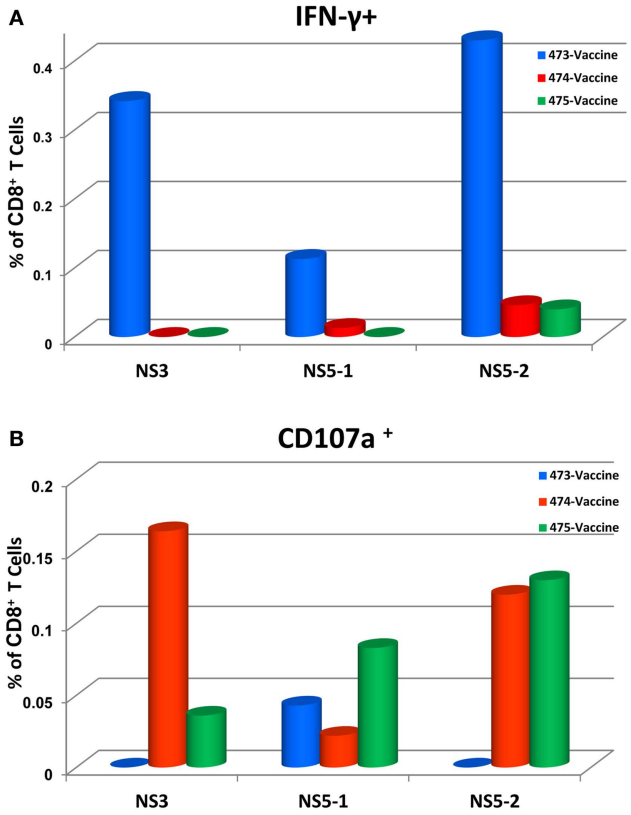

FIGURE 3 | Tetravalent dengue vaccine elicits CD8⁺ IFN-γ producing T cells that cross-react with NS3 and NS5 proteins of DENV-4 (A) and express the CD107a⁺ marker (B). Responses are shown as percentage of T cells from DENV-4 peptide arrays stimulated PBMCs with the background percentage of positive T cells in medium only treated cells subtracted. Peptide arrays for NS5 were split into two pools; NS5-1 and NS5-2. At the time of PBMC testing, peptides arrays for DENV-4 NS1 protein were not available.

The protective efficacy of TDV was assessed against challenge with DENV-2 NGC. All vaccinated animals were protected against DENV-2 NGC as shown by the lack of viral RNA post-challenge, whereas control animals were positive for viral RNA. At the time of challenge, animals from all treatment groups had high levels of anti-DEN-2 neutralizing antibodies (GMT > 300) and their titers were not boosted following challenge. This suggests that the vaccine elicited sterilizing immunity against DENV-2 NGC. Although this study was designed to measure efficacy of TDV using a short-term immunization and challenge protocol, we currently plan to address the longevity of the neutralizing antibody response to vaccination and its impact on protection against challenge with all DENV serotypes. Moreover, the recently published data of the first clinical proof-of-concept efficacy study of a TDV demonstrated safety but only partial efficacy against some but not all DENV viruses, and showed that the standard plaque reduction neutralization test used as the primary immune correlate failed to predict efficacy (23). Therefore, further studies are needed to measure neutralization using different cell substrates (24, 25). Despite the limitations of the NHP model to mimic human disease, efficacy studies based on the presence of viremia as an end point can provide critical information about the protective capacity of candidate DENV vaccines since there are several lines of evidences supporting the view that the severity of disease correlates with increased levels of viremia (26, 27). In conclusion, the delivery of our live-attenuated TDV using the PhJ needle-free DSJI technology has the potential to impact future mass vaccination campaigns.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: Dan T. Stinchcomb, Jorge E. Osorio, Aurelia A. Haller, Charalambos D. Partidos, and Claire Y.-H. Huang. Performed the experiments: Yuping Ambuel, Ginger Young, Joanna Paykel, Kim L. Weisgrau, Michael Royals, and Joseph N. Brewoo. Managed the NHP facility: Saverio Capuano. Analyzed the data: Yuping Ambuel, Eva G. Rakasz,

Table 5 | Protection of TDV vaccinated NHPs from DENV-2 NGC challenge.

Vaccination regimen	Animal ID	Pre-challenge neutralizing titer (day 88)	Post-challenge neutralizing titer (day 108)	Post-challenge viremia (log_{10} copies/ml)					
		Anti-DEN-2 Abs		Day 1	Day 3	Day 5	Day 7	Day 9	Day 11
TDV PhJ/ID (day 0, 0)	CY0503	5120	5120	–	–	–	–	–	–
	CY0504	2560	1280	–	–	–	–	–	–
	CY0505	640	640	–	–	–	–	–	–
TDV PhJ/SC (day 0, 0)	CY0473	1280	2560	–	–	–	–	–	–
	CY0474	320	640	–	–	–	–	–	–
	CY0475	320	320	–	–	–	–	–	–
TDV PhJ/SC (day 0, 60)	CY0493	2560	2560	–	–	–	–	–	–
	CY0494	640	640	–	–	–	–	–	–
	CY0495	2560	2560	–	–	–	–	–	–
PBS PhJ/ID (day 0, 60)	CY0479	5	2560	–	4.8	4.9	4.8	–	–
	CY0481	5	640	–	4.1	4.9	4.9	3.7	4.7
	CY0488	5	1280	–	4.5	5.8	5.2	–	–

Kim L. Weisgrau, and Charalambos D. Partidos. Wrote the paper: Charalambos D. Partidos.

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Conflict of Interest Statement: Yuping Ambuel, Ginger Young, Joseph N. Brewoo, Joanna Paykel, Aurelia A. Haller, Dan T. Stinchcomb, Charalambos D. Partidos, and Jorge E. Osorio are affiliated with Takeda Vaccine, Inc., Michael Royals is affiliated with PharmaJet, Inc. The other authors declare no conflicts of interest.

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Erratum: A rapid immunization strategy with a live-attenuated tetravalent dengue vaccine elicits protective neutralizing antibody responses in non-human primates

Frontiers Production Office*

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Keywords: dengue, vaccine, non-human primates, neutralizing antibodies, needle-free delivery, T cell responses

An erratum on

A rapid immunization strategy with a live-attenuated tetravalent dengue vaccine elicits protective neutralizing antibody responses in non-human primates
*by Ambuel Y, Young G, Brewoo JN, Paykel J, Weisgrau KL, Rakasz EG, Haller AA, Royals M, Huang CY-H, Capuano S, Stinchcomb DT, Partidos CD and Osorio JE. Front Immunol (2014) **5**:263. doi: 10.3389/fimmu.2014.00263*

Reason for Erratum:
In Table 4 the text of subheadings was changed due to a typesetting error. This error does not change the scientific conclusions of the article in any way. The publisher apologizes for this error and the correct version of Table 4 appears below.

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Table 4 | Kinetics of neutralizing antibody responses in animals vaccinated with TDV SC or ID using the PharmaJet device.

NHP ID	Vaccine regimen	Day 30				Day 53				Day 88			
		D1	D2	D3	D4	D1	D2	D3	D4	D1	D2	D3	D4
CY0503	0,0	40	1280	160	20	160	1280	40	10	80	5120	20	40
CY0504	PhJ/ID	80	640	40	10	40	2560	40	5	80	2560	20	40
CY0505		320	1280	2560	320	160	640	640	160	80	640	160	40
	GMT*	*101*	*1016*	*254*	*40*	*101*	*1280*	*101*	*20*	*80*	*2032*	*40*	*40*
CY0473	0,0	2560	10240	160	40	640	5120	80	20	320	1280	40	20
CY0474	PhJ/SC	1280	320	1280	160	640	640	640	160	320	320	160	80
CY0475		640	320	640	320	640	320	320	80	160	320	160	160
	GMT	*1280*	*1016*	*508*	*127*	*640*	*1016*	*254*	*64*	*254*	*508*	*101*	*64*
CY0493	0,60	160	2560	160	20	80	2560	10	20	320	2560	80	40
CY0494	PhJ/SC	320	640	640	160	320	640	320	10	80	640	160	80
CY0495		640	2560	320	40	640	2560	40	80	1280	2560	160	40
	GMT	*320*	*1613*	*320*	*50*	*254*	*1613*	*50*	*25*	*320*	*1613*	*127*	*50*

GMT = geometric mean titer.*