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Polytopic vaccination with a live-attenuated dengue vaccine enhances B-cell and T-cell activation, but not neutralizing antibodies

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Abstract

Dengue, caused by dengue viruses (DENVs), is the most common arboviral disease of humans. Several dengue vaccine candidates are at different stages of clinical development and one has been licensed. Inoculation with live-attenuated DENV constructs is an approach that has been used by vaccine developers. Unfortunately, the simultaneous injection of all four attenuated DENV serotypes (DENV1-4) into a single injection site (monotopic vaccination) has been postulated to result in interference in the replication of some serotypes in favor of others, an important obstacle in obtaining a balanced immune response against all serotypes. Here, we

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demonstrate the virus replicative and immunostimulatory effects of polytopic monovalent dengue vaccination (PV) in which, each of the four components of the tetravalent vaccine is simultaneously delivered to four different sites versus the more traditional monotopic tetravalent vaccination (MV) in a non-human primate (NHP) model. With the exception of DENV-2, there was no significant difference in detectable viral RNA levels between PV and MV inoculation. Interestingly, longer periods of detection and higher viral RNA levels were seen in the lymph nodes of NHPs inoculated PV compared to MV. Induction of lymph node dendritic cell maturation and of blood T- and B-cell activation showed different kinetics in PV inoculated NHPs compared to MV. The MV inoculated group showed earlier maturation of dendritic cells and activation of B and T cells compared to PV inoculated NHPs. A similar kinetic difference was also observed in the cytokine response: MV induced earlier cytokine responses compared to PV. However, similar levels of DENV neutralizing antibodies were observed in PV and MV NHPs. These findings indicate that cellular immune response after vaccination may be affected by the location of inoculation. Design of vaccine delivery may need to take into account the effects of locations of vaccine delivery of multiples serotype live viral vaccine on the induction of immune response.

Keywords: Vaccine, Virology, Immunology, Health sciences

1. Introduction

Dengue virus (DENV) is a mosquito-borne virus of the genus Flavivirus. Dengue disease is a global health problem estimated by the WHO to have 50–100 million DENV infections per year [1]. A more recent estimate placed the number at up to 390 million infections annually, of which 96 million were symptomatic [1]. There are four antigenically distinct DENV serotypes, DENV1-4. Infection with one DENV serotype typically induces lifelong protection against homotypic infection and temporary protection against heterotypic infections [2]. Clinical presentation of DENV infection varies from asymptomatic infection to a non-specific febrile illness (Dengue fever, DF) and the more severe dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Risk for severe dengue disease is associated with a heterotypic secondary DENV infection.

The Sanofi Pasteur tetravalent dengue vaccine (Dengvaxia) recently completed phase III clinical trials in Asia and Latin America. The vaccine demonstrated modest protection (56.5% overall vaccine efficacy) and obtained regulatory approval in several DENV endemic countries [3]. Among the four DENV serotypes that Dengvaxia targets, the vaccine had the lowest efficacy against DENV-2 (35% efficacy) and the highest against DENV-4 (75% efficacy) [3]. These discrepancies in protection may be grounded in an imbalance in the immune responses against the four DENV serotypes. The vaccine, when administered in its

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separate monovalent components, had higher seroconversion rates than when administered in tetravalent formulations, as was shown during pre-clinical studies measuring the vaccine generated DENV-2 neutralizing antibody levels (CYD1-4) [4]. Interference among DENV serotypes administered in the tetravalent formulation is a potential explanation for the apparent reduction in antibodies levels [5].

Interference among viruses delivered together into a single site is a phenomenon previously described in other vaccines. Reduced responses to yellow fever, measles and vaccinia were reported when all three vaccines were delivered together [6]. Similarly, immune responses to varicella vaccines was diminished when it was delivered in combination with mumps, measles and rubella (MMR vaccine), requiring an increase in the varicella component of the vaccine [7, 8]. An example similar to the tetravalent dengue vaccine can be found in polio vaccines. It was found that seroconversion rates to monovalent polio vaccination exceeding 90% were reduced to 50–75% for serotypes 1 and 2 when combined in a trivalent vaccination. Serotype 3 consistently dominated the other two serotypes in secretion, seroconversion and geometric mean titers. Overcoming this imbalance required 3 doses of the vaccine [9]. Earlier live tetravalent dengue vaccines encountered similar limitations. Monovalent dengue vaccines developed by Mahidol University and Aventis Pasteur (now Sanofi Pasteur) demonstrated better titer responses than when formulated in bivalent or trivalent vaccines. In tetravalent formulations, DENV-2 and DENV-4 consistently generated lower titers than monovalent formulation did [10].

Cellular immune responses are as critical as humoral immunity in imparting protection against DENV infection. Virus-induced cellular immune responses are likely dependent on the recognition of cognate T cell populations. Despite the multitude of peptides generally encoded by viruses, peptide immunodominance depends on the ability of cognate T cells to recognize the most immunodominant epitopes [11]. It is possible that when combined into a single formulation, peptides present in all four DENV serotypes compete for recognition by the host T cells, causing disparities in the preference in which they are taken up and processed. This interference, or immunodominance, among DENV serotypes might be reduced by simultaneously inducing T cell responses to dominant epitopes of each dengue serotype.

Computer modeling has shed light on the impact of evolving epitope sequences on immunodominance. Multisite or polytopic injection of different epitopes to areas of the skin with access to different lymph nodes likely reduces immunodominance and increases recognition of the four DENV serotypes [12]. Similar studies addressing multi-site vaccination for cancer [13] describes immune system dynamics that suggest polytopic vaccination leads to more robust immune

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responses than monotopic vaccination. In this work, strains or allele mutants were loaded into T cells located in different lymph nodes which have enough numbers of complementary T cell receptors (TCRs) to proliferate and generate an immune response to such allele.

This study investigates the effects of the simultaneous delivery of each of four DENV serotypes at different injection sites (polytopic vaccination or PV) of a live-attenuated DENV (LAV) vaccine candidate in non-human primates (NHPs) and compared it to the tetravalent delivery of all four serotypes at one injection site (monotopic vaccination or MV). The propagation of four DENV serotypes after MV or PV in serum, saliva and draining lymph nodes was measured. Cell-mediated immunity and neutralizing antibodies were also measured as well as cytokine secretion.

2. Materials and methods

2.1. Animals

Dengue vaccinations were performed in rhesus macaques (*Macaca Mulatta* of Indian origin, both male and female, aged 7–12 years and of 5.8–14 kg of body weight) bred and maintained at the Department of Veterinary Medicine, United States Army Medical Directorate-Armed Forces Research Institute of Medical Sciences (USAMD-AFRIMS, Bangkok, Thailand) under good animal welfare conditions. The USAMD-AFRIMS Animal Care and Use Program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International (AAALAC). Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 2011 edition. The protocol was reviewed and approved by the AFRIMS Institutional Animal Care and Use Committee.

2.2. Dengue vaccine

The live-attenuated DENV vaccine used for MV or PV included the DENV-1 (West Pac74), DENV-2 (S16803), DENV-3 (CH53489) and DENV-4 (TVP360) strains and was manufactured by the Salk Institute for Biological Studies and tested at the Walter Reed Army Institute of Research (WRAIR) [14, 15]. Each of DENV serotype was diluted with MEM medium (Gibco, USA) to make a final concentration of 2×10^5 PFU/ml.

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2.3. Vaccine immunization

Nine monkeys were separated into three groups (2 for negative control, 3 for PV and 4 for MV). For PV, NHPs were subcutaneously vaccinated (0.5 ml) on day 0 with 10^5 PFU of each DENV serotype in their right arms (DENV-1), left arms (DENV-2), right legs (DENV-3) and left legs (DENV-4). In the MV group, the tetravalent dengue vaccine was prepared by mixing all serotypes together (10^5 PFU/each serotype) and NHPs were subcutaneously vaccinated in a single site (right arm). Blood was collected from each group of vaccinated NHPs each day from day 0 to day 15 and again on day 30. Individual serum samples were used to determine the RNA level, DENV neutralizing antibodies and cytokine/chemokine production. Saliva was collected daily for 15 days after vaccination to measure viral load in the oral cavity. Four lymph nodes (left and right axillary; left and right inguinal) were collected from one half of each group on days 5 and 15; and from the other half on days 10 and 20, to measure dengue viral RNA and cell-mediated immune responses. Only one NHP in the PV group was available for the day 10 and day 20 lymph node collection.

2.4. Biosamples

Blood and saliva were collected daily from anesthetized NHPs from day 0 to 15 post vaccination. The levels of viral RNA were determined by qRT-PCR. Sera collected on days 0, 15, and 30 were used to measure DENV neutralizing antibody by PRNT. Cytokine production in NHPs was also determined in serum on days 0, 1, 3, 5, 7, 9, 11, and 15. One right and one left axillary lymph node and one right and one left inguinal lymph node were collected on days 5, 10, 15, and 20. Lymph nodes were processed into single-cell suspensions. Half of the suspension was used for viral RNA level determination and the other half for cellular immune responses by flow cytometry.

2.5. Quantitative real-time RT-PCR for DENV

Viral RNA was extracted from 140 μl of serum, saliva and LN single-cell suspension by using QIAamp viral RNA mini kit (QIAGEN, Germany) according to the manufacturer's instruction. Viral RNA from the lymph node specimen was extracted by using RNeasy Mini kit (QIAGEN, Germany) according to the manufacturer's instruction. DENV quantitative real-time RT-PCR (qRT-PCR) was performed according to the method described by Sadon et al., with the following modifications [16], One-Step RT-PCR kit (Ambion, Austin, TX, USA) was used. Two separate duplex reactions were carried out for each RNA sample; one for simultaneous detection of DENV-1 and DENV-3 and the other for DENV-2 and DENV-4. DENV-1/DENV-3 reaction mixture contained 2.5 μl RNA template (corresponding to approximately 400 ng RNA/sample), 10 pmol of each DENV-1

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and DENV-3 primers, 5 pmol of each DENV-1 and DENV-3 probes, 1.67 μl of Detection Enhancer, 20 U of RNAsin, 1X RT-PCR buffer and 1X RT-PCR enzyme mix, in a total volume of 25 μl. DENV-2/DENV-4 reaction mixture contained the same composition except that DENV-2/DENV-4 primers and probes were used. Primer/probe sequences previously described by Sadon et al., 20007 were used except for the DENV-1 primers and probe. DENV-1 forward and reverse primers and probe used in this study were TMF; 5′-GCATTYCTAAGATTTCTAGCCA-TACC-3′, TMR; 5′-TCGCTCCATTCTTCTTGAATGAG-3′, and TMP; 5′-AACAGCAGGAATTTT-3′ (5′-FAM/3′-MGB), respectively. The single-step RT-PCR consisting of a 10-min RT step at 45 °C, 10 min of Taq polymerase activation at 95 °C, followed by 40 cycles of PCR at 95 °C for 15 sec and 60 °C for 1 min was performed on an ABI 7300 Real Time PCR system (ABI).

2.6. Dengue neutralizing antibody

To determine the immunogenicity of PV and MV, we measured neutralizing (NT) antibodies in each individual serum sample against DENV-1 (16007), DENV-2 (16681), DENV-3 (16562) and DENV-4 (C0036/06) by plaque reduction neutralization test ($PRNT_{90}$) [17]. The assay was done in three independent experiments (in duplicate wells, each experiment). Serum was diluted in a four-fold serial dilution starting from 1:10 to 1:2,560 (terminal dilution) and mixed with the virus in equal volumes before incubation in a 35 °C water bath for 1 h. The mixture was inoculated into Rhesus monkey kidney epithelial cells (LLC-MK2 cells) in a 12-well plate and incubated at room temperature (RT) for 1 h on a rocker platform. The excess volume of the inoculum was removed. Medium over layer containing low melting point agarose gel (LMP, Ultra Pure^{TM} LMP agarose, Invitrogen, USA) was then added. DENV-infected cells were incubated for 4–6 days in a 5% CO_2 incubator at 35 °C before staining with 4% neutral red (Sigma, USA) in a second medium over layer with LMP. The number of plaques was determined by manual counting. NT antibody titer was calculated by SPSS program, using regression with probit analysis at 50% reduction which was identified as the highest dilution that can reduce the number of plaques by 50% as compared to the number of plaques in the virus control well.

2.7. Cell-mediated immune responses

Lymph nodes (left/right axillary and left/right inguinal) were collected and pushed through a mesh cell strainer to obtain a single cell suspension. Single cell suspensions from each lymph node were separated into two parts, one for qRT-PCR and another for cell-mediated immunity (CMI) testing. Anti-NHP antibodies used to stain the LN cells included anti-CD3 (clone SP34-2), anti-CD4 (Clone L-200), anti-CD8 (Clone RPA-T8), anti-CD20 (clone 2H7), anti-CD69 (Clone FN-50), anti-CD11c (Clone S-HCL-3), anti-CD80 (Clone L307.4), and anti-CD86

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(Clone 2331) (BD Pharmingen). Each of the antibodies was added into the cell suspension using a dilution of 1:50 and incubated at 4 °C for 30 min. The stained cells were then washed three times with BD perm wash (BD Biosciences). The frequency of cell marker expression was measured by BD LSRFortessa^{TM} Cell Analyzer (BD Biosciences). Twenty-thousand events were collected to calculate the positive frequency.

2.8. Cytokine production

Collected serum samples were tested individually for the presence of IL-1β, IL-1ra, IL-6, IL-12, interferon (IFN)-γ, MIP-1α, and TNF-α using the MILLIPLEX Non-Human Primate Cytokine-Chemokine Array kit (Millipore) following the manufacturer's instruction. The assay was performed in triplicate wells. Briefly, dye bead-coupled capture antibodies were incubated with standards or serum samples for 30 min on an 800 rpm shaker at room temperature. Unbound material was washed prior to incubation with biotinylated detection antibodies for 30 min on an 800 rpm shaker at room temperature. After washing away unbound biotinylated antibodies, a reporter streptavidin-phycoerythrin conjugate was added to the beads and incubated for 10 min on an 800 rpm shaker at room temperature. After removing excess streptavidin-phycoerythrin, the bound beads were counted via a dual laser flow-based reader, which measures the fluorescence of the bound SA-PE in terms of mean fluorescence intensity (MFI).

2.9. Statistical analysis

Mann-Whitney U test/Kruskal-Wallis analysis was used to compare the differences of RNA levels in lymph node, viral load in saliva, neutralizing antibody level and cytokine productions from P V and MV. A p value less than 0.05 was considered statistically significant.

3. Results

3.1. Quantitative RT-PCR of dengue RNA levels in blood, saliva and lymph nodes

Collected sera from control, PV and MV groups were used to determine RNA levels of four DENV serotypes by qRT-PCR. The results suggest that MV inoculation could generate longer periods of viral RNA for all DENV serotypes than PV inoculation (Table 1). Dengue RNA in the serum of the MV group ranged from 3.25 days for DENV-4 to 10.25 days for DENV-1, while PV inoculation yielded periods of serum viral RNA ranging from 1.67 days for DENV-3 to 9.33 days for DENV-1. However, these differences were not significant. The onset of serum viral RNA post inoculation appears to occur sooner in the PV group (from one day post inoculation for DENV-1 and 3 days post inoculation for DENV-4)

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Table 1. Comparison of dengue viral RNA level in serum, saliva, and lymph nodes (LNs) of control, PV and MV groups.

		Control	PV	MV
Serum Mean Number of viral RNA (days)	DENV-1	0	9.33	10.25
	DENV-2	0	4.67	7.25*
	DENV-3	0	1.67	3.75
	DENV-4	0	2.67	3.25
Serum Mean viral RNA (GE/ml)	DENV-1	BLOD	2.2×10^4	1.5×10^4
	DENV-2	BLOD	3.5×10^3	7.5×10^2
	DENV-3	BLOD	9.2×10^2	4.0×10^3
	DENV-4	BLOD	1.8×10^3	6.1×10^3
Serum Mean Day Onset of viral RNA	DENV-1	N/A	1	1.25
	DENV-2	N/A	1	2
	DENV-3	N/A	2	4.5
	DENV-4	N/A	3	1
Saliva Mean Number of viral RNA (days)	DENV-1	0	0.7	1
	DENV-2	0	0	0
	DENV-3	0	0.3	0
	DENV-4	0	0	0
Saliva Mean viral RNA(GE/ml)	DENV-1	BLOD	2.0×10^2	1.1×10^2
	DENV-2	BLOD	BLOQ	BLOQ
	DENV-3	BLOD	1.9×10^3	BLOQ
	DENV-4	BLOD	BLOQ	BLOQ
% LN viral RNA Positive Days (Mean % of Positive LN per NHP)	DENV-1	0 (0)	100 (83)	68 (50)
	DENV-2	0 (0)	83 (71)	48 (31)
	DENV-3	0 (0)	33(8)	12 (3)
	DENV-4	0 (0)	0 (0)	14 (8)
LN Mean viral RNA (GE/ml)	DENV-1	BLOD	3.3×10^2*	9.8
	DENV-2	BLOD	2.3×10^2*	37
	DENV-3	BLOD	8.8	BLOQ
	DENV-4	BLOD	BLOQ	2.3×10^2*

The Mean Number of viral RNA (days) is the average number of the days that viral RNA can be detected in the serum. The "% LNs viral RNA Positive Day" is the day that viral RNA can be detected in each LNs from each group. BLOD: Below Limit of Detection. BLOQ: Below Limit of Quantification (viral RNA lower than 5 GE/ml). The symbol (*) indicates a significant difference of $p \leq 0.05$ between PV and MV.

than in the MV group (from 1 day post inoculation for DENV-4 to 4.5 days for DENV-3). Despite the differences in the number of DENV-2 viral RNA positive days and the onset of viral RNA, no significant differences in viral RNA levels

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were noted (as measured in GE/ml, Table 1). We further investigated the present of DENV RNA in saliva. However, only a low amount of DENV-1 was detected in both PV and MV groups whereas DENV-3 was detectable only in PV group. In an attempt to quantify DENV RNA in the lymph nodes (LNs), we harvested 4 LNs (see methods) from each inoculated NHP in alternate days (either in Days 5 and 15 or Days 10 and 20 post inoculation). NHP in the PV group displayed DENV viral RNA in at least one LN in a higher percentage of sampled days than in the MV group for DENV-1 trough DENV-3, but not DENV-4 (Table 1 and Table 2). Further, we determined that the percentage of positive LNs (Table 1, in parenthesis) in each NHP during DENV-1 through DENV-3 positive days was higher in the PV group as well. Similarly, the PV group had, with the exception of DENV-4, higher viral RNA levels ($p < 0.05$ for DENV-1 and DENV-2) than the MV group (Table 1). Interestingly, only in the PV group were we able to detect any viral RNA on Day 20 post-inoculation (for DENV-1 and DENV-2), which was longer that viral RNA found in the serum (Table 2).

3.2. Dengue neutralizing antibody

Vaccine-induced NT levels against all four DENV serotypes were measured by $PRNT_{90}$ on serum collected on days 0, 15, and 30 post inoculation (Fig. 1). There were no detectable NT antibodies in the control animal at any time. Both MV and PV groups generated NT antibody titers against all DENV serotypes on days 15 and 30, and no significant decline of NT antibodies against any of the DENV serotypes was observed after day 15 post inoculation. Generally, $PRNT_{90}$ titers against DENV-1 and DENV-2 were higher than against DENV-3 and DENV-4. With few exceptions, PV- and MV-induced $PRNT_{90}$ titers were similar. By day 30, the MV group generated significant higher $PRNT_{90}$ titers ($p \leq 0.05$) against DENV-4 than PV did.

3.3. Cell-mediated immune response

We determined the frequencies of activated B and T cells in the axillary and inguinal LNs from control and vaccinated NHPs collected on days 5, 10, 15 and 20 post inoculation by flow cytometry. Fig. 2 shows the frequencies of B and T cells expressing the activation marker CD69 in the lymph nodes of these animals. LN from PV immunized animals contained significantly higher frequencies of $CD20^+$ B-cells expressing CD69 on day 10 and 15 as compared to the MV group and on day 20 as compared to all groups. The frequency of $CD3^+$ T cells expressing CD69 in the PV group was significantly higher on day 20 as compared to the MV group (Fig. 2, upper left and upper right panels). Interestingly, MV was associated with an earlier activation of T cells (as compared to the PV group) as indicated by the frequencies of CD69 expressing cells at the earliest time point post inoculation (day 5). PV was also associated with significantly higher frequencies of CD3

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Table 2. Comparison of dengue viral RNA level in lymph nodes (LNs) of control, PV and MV groups.

Group	Monkey		DENV-1				DENV-2				DENV-3				DENV-4			
		Specimen	Day 5	Day 10	Day 15	Day 20	Day 5	Day 10	Day 15	Day 20	Day 5	Day 10	Day 15	Day 20	Day 5	Day 10	Day 15	Day 20
Control	R130	Left Arm	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A
		Right Arm	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A
		Left Leg	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A
		Right Leg	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A
	R106	Left Arm	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD
		Right Arm	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD
		Left Leg	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD
		Right Leg	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD
PV	R630	Left Arm	BLOD	N/A	BLOD	N/A	52.4	N/A	493.5	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A
		Right Arm	196.6	N/A	153.6	N/A	89.8	N/A	54.9	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A
		Left Leg	BLOD	N/A	372.0	N/A	66.3	N/A	243.1	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A
		Right Leg	133.1	N/A	402.2	N/A	BLOD	N/A	32.5	N/A	109.8	N/A	101.4	N/A	BLOD	N/A	BLOD	N/A
	R307	Left Arm	N/A	62.8	N/A	168.6	N/A	417.8	N/A	927.6	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD
		Right Arm	N/A	242.0	N/A	123.6	N/A	BLOD	N/A	108.1	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD
		Left Leg	N/A	12.5	N/A	179.6	N/A	64.2	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD
		Right Leg	N/A	33.0	N/A	191.2	N/A	1815.7	N/A	55.8	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD
	R707	Left Arm	N/A	194.2	N/A	54.1	N/A	BLOD	N/A	109.2	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD
		Right Arm	N/A	57.7	N/A	BLOD	N/A	152.7	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD
		Left Leg	N/A	226.4	N/A	154.6	N/A	750.1	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD
		Right Leg	N/A	805.3	N/A	154.9	N/A	247.9	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD
MV	R420	Left Arm	46.7	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	1108.3	N/A	BLOD	N/A
		Right Arm	14.0	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	355.8	N/A	BLOD	N/A
		Left Leg	12.7	N/A	BLOD	N/A	65.1	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	719.4	N/A	BLOD	N/A

(*Continued*)

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Table 2. (*Continued*)

Group	Monkey		DENV-1				DENV-2				DENV-3				DENV-4			
		Specimen	Day 5	Day 10	Day 15	Day 20	Day 5	Day 10	Day 15	Day 20	Day 5	Day 10	Day 15	Day 20	Day 5	Day 10	Day 15	Day 20
		Right Leg	12.0	N/A	95.3	N/A	101.7	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A
	R616	Left Arm	BLOD	N/A	192.3	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	1116.9	N/A	BLOD	N/A
		Right Arm	209.7	N/A	293.3	N/A	912.3	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	3477.7	N/A	BLOD	N/A
		Left Leg	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A
		Right Leg	28.2	N/A	115.4	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	213.6	N/A	BLOD	N/A
	R544	Left Arm	N/A	22.9	N/A	BLOD	N/A	45.8	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD
		Right Arm	N/A	231.7	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD
		Left Leg	N/A	108.0	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD
		Right Leg	N/A	137.9	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	222.6	N/A	BLOD
	R626	Left Arm	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD
		Right Arm	N/A	48.6	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD
		Left Leg	N/A	71.4	N/A	30.3	N/A	BLOD	N/A	62.9	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD
		Right Leg	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD	N/A	BLOD

BLOD: Below Limit of Detection. BLOQ: Below Limit of Quantification (viral RNA lower than 5 GE/ml).

N/A: Not applicable.

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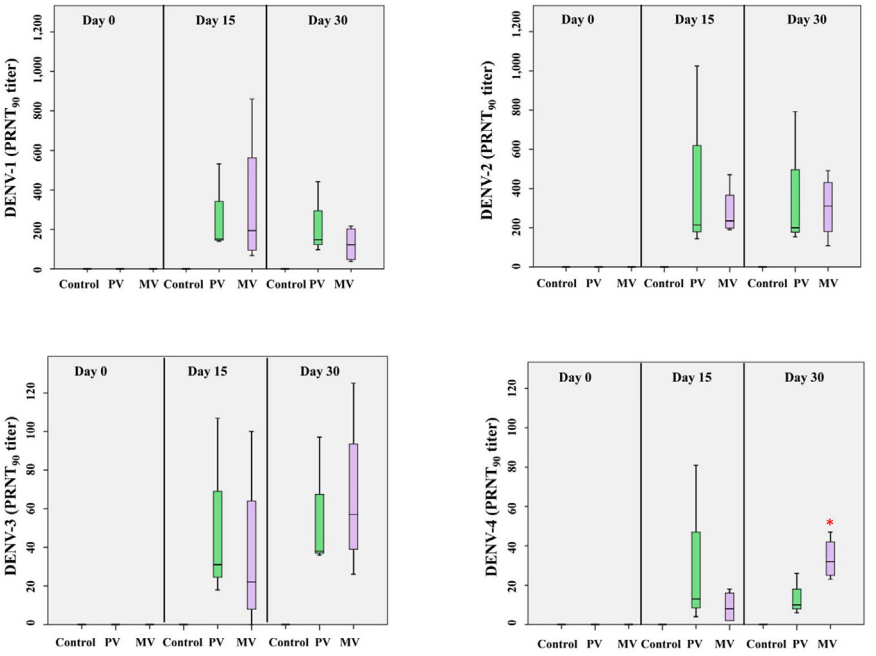

Fig. 1. Dengue neutralizing antibody levels against four DENV serotypes were determined by $PRNT_{90}$ on serum collected on days 0, 15, and 30 post inoculation from control, MV and PV groups.

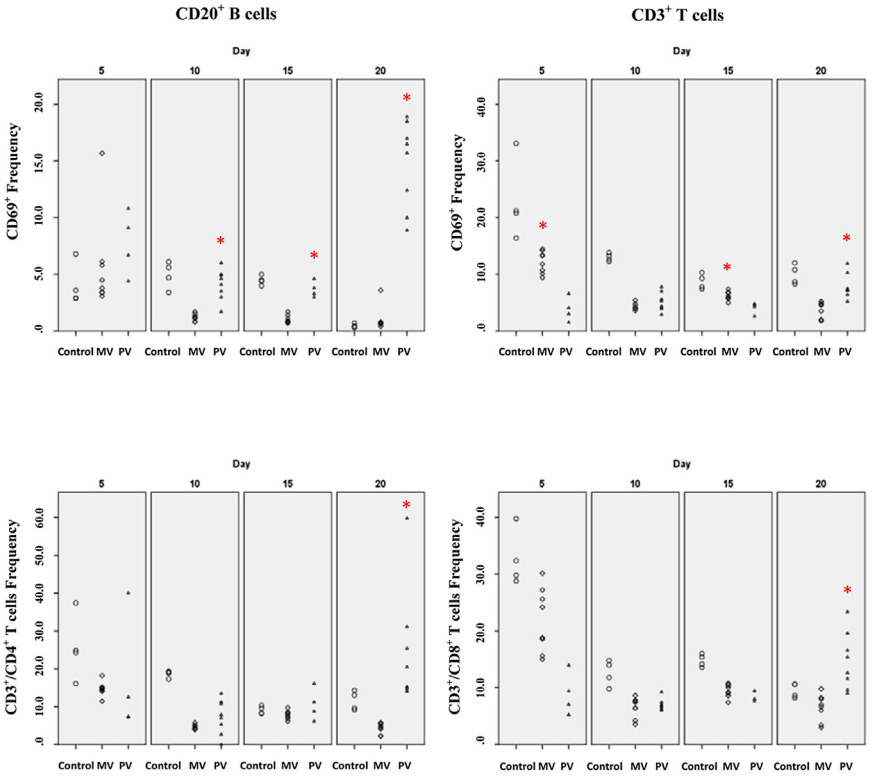

Fig. 2. Cell-mediated immunity induced by MV and PV.

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$^{+}$/CD4^{+} and CD3^{+}/CD8^{+} T-cells in the lymph nodes by day 20 (Fig. 2, lower left and lower right panels). Analysis of the expression of the costimulatory molecules CD80 and CD86 on CD11c^{+} dendritic cells revealed distinct patterns in MV and PV groups respectively (Fig. 3). MV induced higher levels of CD80 surface expression as compared to the PV group. This was most significant by day 5, which then normalized by day 20 post inoculation. PV induced higher levels of CD86 surface expression than the MV group through day 20 post inoculation.

3.4. Cytokine and chemokine production

We quantified the levels of inflammatory cytokines in the serum of control, MV and PV groups on days 0, 1, 3, 5, 7, 9, 11, and 15 post inoculations (Fig. 4, Table 3). In general, MV was associated with higher levels of serum cytokines.

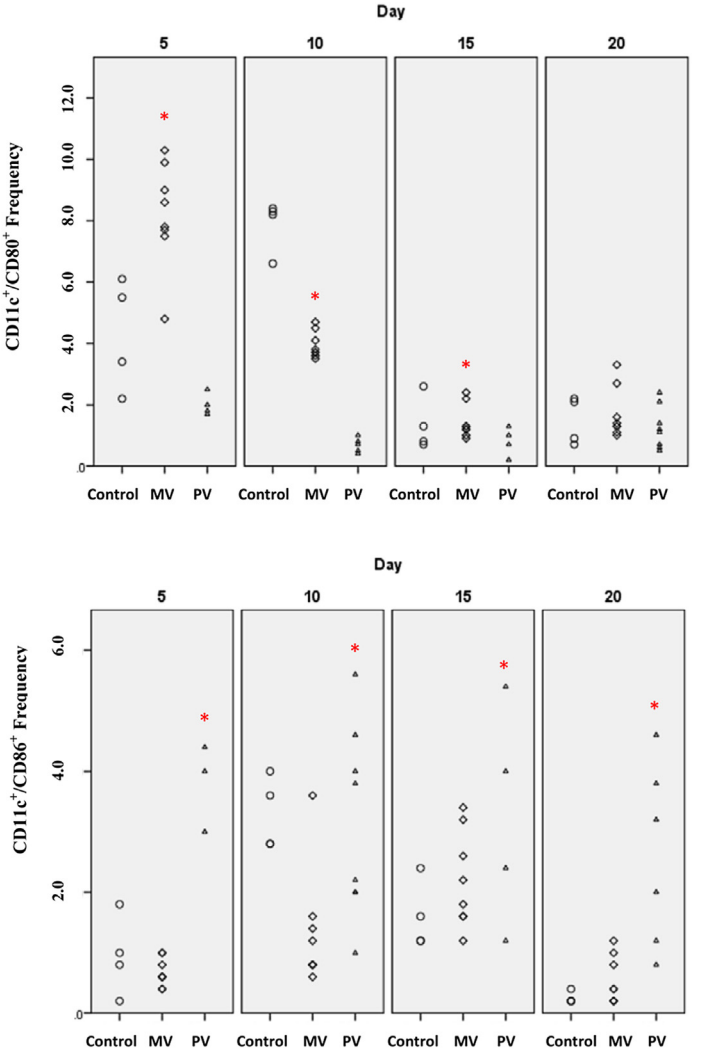

Fig. 3. Dendritic cell maturation markers-induced by MV and PV.

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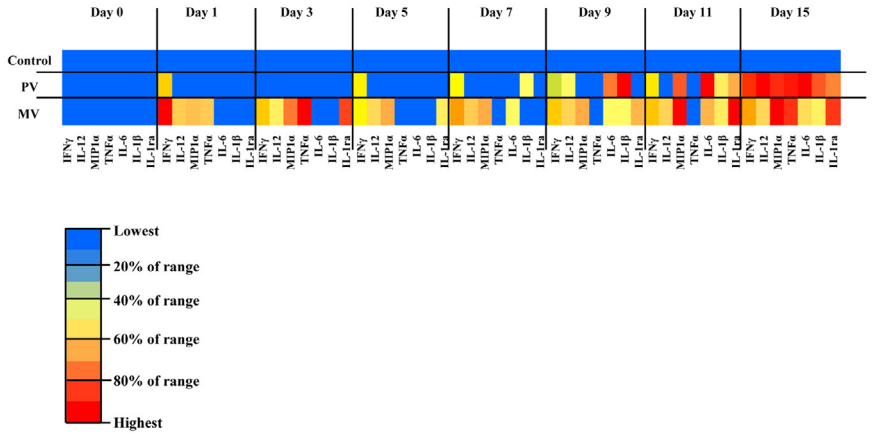

Fig. 4. Heat map displays cytokines and chemokines production in serum of control, PV and MV groups determined by Bio-plex assay.

IFNγ, IL-12, and MIP-1α were detected in the serum of MV-inoculated NHPs early after inoculation and through day 15. MV also induced higher level of TNFα, IL-6, IL-1β, and IL-1Ra secretion, but at later time points (Day 5 and day 7 post-inoculation). PV inoculation had a more delayed effect, inducing secretion starting at day 9 of the pro-inflammatory cytokines IL-1β and IL-6, at significantly ($p \leq 0.05$) higher levels than the control and MV groups. A different expression pattern was observed with IFN-γ (Th1 type cytokine) production; MV generated higher IFN-γ early after inoculation than PV (Day 3). However PV induced higher levels of IFN-γ at later time points (Day 15). MIP-1α showed a similar pattern, with MV inducing early MIP1α while PV induced MIP-1α production later.

4. Discussion

The efficacy of Sanofi Pasture's tetravalent dengue vaccine, Dengvaxia, varied in recent phase III trials from only about 35% against DENV-2 to 75% against DENV-4. Many factors may be responsible for this phenomenon, including convalescence status and age of the subjects. Among these factors is the potential imbalance in vaccine immune responses against the four DENV serotypes [3]. This imbalance may be a reflection of the interference among the viruses during replication and immune induction [5]. Incomplete protection delivered by a tetravalent dengue vaccine raises concerns of antibody-dependent enhancement of infection [18]. The preferred method of delivering all DENV serotypes into a single injection site may alter the preference by which important T cell epitopes are targeted and the corresponding T cell responses (original antigenic sin) [19, 20, 21]. It is possible that the simultaneous induction of the host immune cells by polytopic delivery of each DENV serotype dominant epitope could reduce this interference, and hence, improve vaccine efficacy [19, 22].

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Table 3. Cytokine production in the serum of control, PV and MV vaccinated NHPs. Serum samples from each group (Days 0, 1, 3, 5, 7, 9, 11 and 15) were tested individually for the presence of IL-1β, IL-1ra, IL-6, IL-12, interferon (IFN)-γ, MIP-1α and TNF-α using the bead-based MILLIPLEX Non-Human Primate Cytokine-Chemokine Array kit (Millipore). The symbols "*" and "**" indicate significant different ($p < 0.05$) of PV > MV and MV > PV, respectively.

Cytokine	Group	Day 0	Day 01	Day 03	Day 05	Day 07	Day 09	Day 11	Day 15
IFNγ	Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
(pg/ml)	PV	0.0	12 ± 13	0.0	7.2 ± 11.3	5.5 ± 5.3	12 ± 13	12 ± 13	12 ± 13
	MV	0.0	44.7 ± 35.7	13.0 ± 8.7**	6.1 ± 5.1	20.2 ± 20.7	12.8 ± 20.5	13.8 ± 15.1	18.8 ± 25.2
IL-12	Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
(pg/ml)	PV	0.0	0.0	0.0	0.0	0.0	2.6 ± 4.6	0.0	121.6 ± 210.6
	MV	0.0	24.2 ± 55.8	8.1 ± 18.7	18.5 ± 42.8	24.8 ± 57.2	20.6 ± 47.7	21.3 ± 49.1	19.2 ± 44.3
MIP1α	Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
(pg/ml)	PV	0.0	0.0	0.0	0.0	0.0	0.0	70.1 ± 121.4	89.6 ± 155.1
	MV	0.0	26.5 ± 53.0	56.2 ± 112.3	35.4 ± 70.8	35.7 ± 71.4	35.2 ± 70.3	105.9 ± 123.2	107.2 ± 102.2
TNFα	Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
(pg/ml)	PV	0.0	0.0	0.0	0.0	0.0	0.0	0.0	114.9 ± 199.0
	MV	0.0	26.1 ± 52.1	126.8 ± 253.7	0.0	0.0	0.0	0.0	102.1 ± 204.2
IL-6	Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
(pg/ml)	PV	0.0	0.0	0.0	0.0	0.0	131.1 ± 227.0	246.8 ± 275.8*	248.6 ± 322.7*
	MV	0.0	0.0	0.0	0.0	2.2 ± 4.4	4.0 ± 7.9	72.0 ± 97.1	33.4 ± 66.8
IL-1β	Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
(pg/ml)	PV	0.0	0.0	0.0	0.0	1.7 ± 3.0	297.2 ± 301.2*	22.4 ± 14.6	196.3 ± 283.4*
	MV	0.0	0.0	0.0	0.0	0.0	5.6 ± 8.3	22.6 ± 25.6	16.1 ± 19.0
IL-1ra	Control	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
(pg/ml)	PV	0.0	0.0	0.0	0.0	0.0	0.0	20.5 ± 35.5	49.7 ± 86.0
	MV	0.0	0.0	47.1 ± 74.6	4.4 ± 8.9	0.0	18.3 ± 31.7	63.5 ± 82.0	31.0 ± 26.9

The impact of PV vaccination on different lymph nodes has been previously modeled and shows that PV could reduce immunodominance and increase recognition of all DENV serotypes [19]. A follow-up study using a constructed analytical model of immune system dynamics of multi-site vaccination for cancer revealed that PV can lead to a better immune response than MV. In this model, each strain or allele mutants was loaded into T cells in different lymph nodes helping to avoid competition for TCR [23, 24]. This study tentatively demonstrated the importance of PV for live-attenuated tetravalent vaccines.

In our study, a mixture of four live attenuated DENV serotypes were simultaneously injected at the same site in the MV group. This form of inoculation will have all DENV serotypes compete for the target cells, with one virus potentially becoming the most dominant. Alternatively, in PV, each DENV serotype is deposited in a different location from the other three, allowing them to simultaneously infect different cells, potentially reducing interference among DENV serotypes as they compete for target cells. Perhaps due to the low number of NHPs in our studies, we could not observe significant differences in viral RNA levels in the serum, with the exception of DENV-2. However, the longer periods and higher viral RNA levels of viral RNA (DENV-1 to -3) in the LNs as well as differences in the level of cellular activation in the LNs of PV immunized animals suggested PV inoculation was associated with a more prolonged immune induction due to more sustained viral replication.

There were no significant differences in viral RNA levels as they oscillated between 10^2 to 10^4 GE/ml for all serotypes in the two groups (Table 1). However, viral RNA, although at very low levels, could be found at higher frequency in the LNs of the PV group suggesting that DENV had greater accessibility to and replication in LNs of the PV-inoculated NHP. Nonetheless, these apparent discrepancies in viral RNA levels in the two groups did not translate into differences in induced humoral immunity as we detected comparable neutralizing antibody (NT) titers between PV and MV (Fig. 1). Since $PRNT_{90}$ were performed by day 30 post inoculation, this *in vitro* finding of comparable neutralizing antibody may not reflect actual differences in immunity *in vivo*. Testing of PRNT activity at a later time points is needed to evaluate the duration of immunity elicited by the two inoculation methods.

Cell-mediated immunity plays an important role in protection against dengue disease and viral clearance. The inclusion of T-cell epitopes in vaccines has been shown to improve viral clearance in murine and NHP models [25, 26]. It is believed that T cells responses to each serotype are largely due to a dominant single epitope [27, 28]. The T cell original antigenic sin postulate underscores the importance of epitopes selection and the impact on T cell responses [29]. It is possible that simultaneous induction (via PV) of host immunity by dominant

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epitopes of each DENV serotype could reduce the interference among the four DENV serotypes [12]. As such, it is possible that PV and MV inoculation may induce cell-mediated immune responses that behave differently. In our study, we found that the longer persistence of viral RNA in the serum in the PV group (Table 1) correlated with higher frequencies of activated B and T cells (over the MV group) at later time points (Fig. 2). Nonetheless, and confounding the data, we observed high frequencies of activated B and T cells in the control group. The medical records of the NHP in this group did not have any indication (behaviors and general physical examination) that would suggest that these NHP were ill during this study. A potential explanation was offered by work done with the yellow fever virus vaccine, YF-17D. This study shows that after vaccination with YF-17D, most effector T cells undergo apoptosis after the clearance of the YF-17D virus, and that only a small proportion of them differentiate into the viral NS4B protein-specific poly-functional memory T cells that provide long-term protection against viral infection [30]. We speculate that this phenomenon might occur in our study and it may be reflected in the apparent high frequency of activated lymphocytes in control group LNs collected in day 5. It is possible that a deletion event may be taking place in the MV and PV inoculated NHP and as such effector T cells may be undergoing apoptosis when we tested them. Unfortunately, we did not measure dengue-specific T and B cells responses due to the limited amount of LN cells we captured. Similarly, we found that PV inoculation was a stronger inducer of CD86 expression in DCs (Fig. 3, lower panel). While both CD80 and CD86 induce T cell activation [31], CD86 (found in the PV group at higher frequency) has been shown to be a stronger activator. Expression of CD80 (found in the MV group at higher frequency than in the PV group) is thought to play a role in self-tolerance.

Taken together, and with the exception of DENV-2, we could not demonstrate significant differences in viral RNA levels, number of viremic days and DENV neutralizing antibody titer between the MV and the PV groups. However, the data suggest that PV inoculation triggers activation of T and B cells as well as DCs in ways that are different to MV. Interestingly, and at least in this case, higher viremia in the serum was not a requisite for higher levels of cellular activation. Increased T and B cell activation and expansion of naïve and memory $CD4^+$ T Cells [32, 33] in the PV group may be reflected in the increased serum levels of IL-1β and IL-12. The inflammasome complex formation might be a sensor potentially activated in the PV group, as levels of IL-1β, a product of inflammasome [34], were higher than in the MV group. Unfortunately, we were unable to obtain sufficient number of cells to conduct cell-mediated immunity assays using *ex-vivo* stimulation with viral antigens.

The increase in immune cell activation induced by PV delivery of a live-attenuated dengue vaccine indicates the potential for PV inoculation as a tool to reduce

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interference among the four DENV serotypes. However, further experiments will need to be performed to further elucidate differences in immune cell activation between PV and MV inoculations. In summary, this study adds additional knowledge on vaccine delivery and further contributes to the development of multi-strain virus vaccines.

Declarations

Author contribution statement

Taweewun Hunsawong: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Sineewanlaya Wichit, Thipwipha Phonpakobsin, Yongyuth Poolpanichupata, Napaporn Latthiwongsakorn: Performed the experiments.

Chonticha Klungthong, Butsaya Thaisomboonsuk, Rawiwan Im-erbsin, Louis R. Macareo: Contributed reagents, materials, analysis tools or data.

In-Kyu Yoon: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Damon W. Ellison: Analyzed and interpreted the data; Wrote the paper.

Anon Srikiatkhachorn: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Robert V. Gibbons, Stefan Fernandez: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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