Research of the long-term immune response in humans has provided several interesting parallels to the data. It was reported that complete cross-protective immunity from heterologous challenge was induced in individuals 1–2 months after a primary DENV infection, with partial immunity present up to 9 months, resulting in a milder disease of shorter duration on reinfection, and that complete serotype-specific immunity against symptomatic dengue was observed up to 18 months postinfection [30]. Guzman and Sierra have previously recorded the long-term presence of both DENV-specific antibodies and T cells up to 20 years after natural infections [10, 31]. Of note, increased T cell activation is reportedly associated with severe dengue disease [7, 8]. Thus, the balance between humoral and cellular immunity may be important in the control of dengue diseases.

However, the details regarding the implication of humoral and cellular immunity in controlling DENV infection remain to be elucidated. Previously, passive transfer of either monoclonal or polyclonal antibodies was shown to protect against homologous DENV challenge [13, 15, 16]. It was also reported that neutralizing antibodies played a greater role than cytotoxic T lymphocyte (CTL) responses in heterologous protection against secondary DENV infection *in vivo* in IFN-$\alpha/\beta R^{-/-}$ and $IFN\gamma R^{-/-}$ mouse models [18]. Moreover, $CD4^+$ T cell depletion did not affect the DENV-specific IgG or IgM Ab titers or their neutralizing activity in the $IFN\gamma R^{-/-}$ mouse model [36]. On the other hand, there are several reports showing that cellular immunity rather than humoral immunity plays an important role in the clearance of DENV. For example, in adoptive transfer experiments, although cross-reactive DENV-1-specific $CD8^+$ T cells did not mediate protection against a lethal DENV-2 infection, adoptive transfer of $CD4^+$ T cells alone mediated protection and delayed mortality in IFN-$\alpha/\beta R^{-/-}$ and $IFN\gamma R^{-/-}$ mouse models [39]. It has also been demonstrated that $CD8^+$ T lymphocytes have a direct role in protection against DENV challenge in the IFN-$\alpha/\beta R^{-/-}$ mouse model of DENV infection by depleting $CD8^+$ T cells [35]. In addition, previous data from adoptive-transfer experiments in BALB/c mice showed that cross-reactive memory $CD8^+$ T cells were preferentially activated by the secondary DENV infection, resulting in augmented IFN-γ and tumor necrosis factor-α (TNF-α) responses, and this effect was serotype-dependent [2, 3]. Although it has previously been suggested that inducing neutralizing antibodies against DENV may play an important role in controlling DENV infection, CTLs are also proposed to contribute to clearance during primary DENV infection and to pathogenesis during secondary heterologous infection in the BALB/c mouse model [4].

Why did the mouse models of DENV infection show inconsistent results *in vivo*? One of the reasons could be that these results were obtained mainly from genetically manipulated mice such as IFN-$\alpha/\beta R^{-/-}$ and $IFN\gamma R^{-/-}$ mice. Moreover, these mice were inoculated with 10^9–10^{10} genome equivalents (GE) of DENV [27, 35, 36], which were likely in large excess compared with the 10^4-10^5 GE of DENV injected into humans by a mosquito [19]. In addition, the efficiency of DENV replication in wild mice *in vivo* is very low compared to that in humans [35].

Recently, novel non-human primate models of DENV infection using rhesus macaques as well as marmosets and tamarins have been developed [24–26, 38]. An intravenous challenge of rhesus macaques with a high dose of virus inoculum (1×10^7 GE) of DENV-2 resulted in readily visible hemorrhaging, which is one of the cardinal symptoms of human DHF [26]. It was also shown that the cellular immune response was activated due to expression of IFN-γ, TNF-α, and macrophage inflammatory protein-1 β in $CD4^+$ and $CD8^+$ T cells during primary DENV infection in rhesus macaques [20]. On the other hand, in the marmoset model of DENV infection, we observed high levels of viremia (10^5–10^7 GE/ml) after subcutaneous inoculation with 10^4–10^5 plaque-forming units (PFU) of DENV-2. Moreover, we demonstrated that DENV-specific IgM and IgG were consistently detected and that the DENV-2 genome was not detected in any of these marmosets inoculated with the same DENV-2 strain used in the primary infection [24]. It is notable that while neutralizing antibody titers were at levels of 1:20-1:80 before the re-challenge inoculation, the titers increased up to 1:160-1:640 after the re-challenge inoculation [24]. These results suggested that the secondary infection with DENV-2 induced a protective humoral immunity to DENV-2 and that DENV-infected marmoset models may be useful in order to analyze the relationship between DENV replication and the dynamics of adaptive immune responses *in vivo*.

Taking these findings into consideration, we investigated the dynamics of cellular immunity in response to primary and secondary DENV infection in the marmoset model.

Materials and methods

Animals

All animal studies were conducted in accordance with protocols of experimental procedures that were approved by the Animal Welfare and Animal Care Committee of the National Institute of Infectious Diseases, Japan, and the National Institute of Biomedical Innovation, Japan. A total of six male marmosets, weighing 258–512 g, were used. Common marmosets were purchased from Clea Japan Inc.

(Tokyo, Japan) and caged singly at 27 ± 2 °C in 50 ± 10 % humidity with a 12-h light-dark cycle (lighting from 7:00 to 19:00) at Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Tsukuba, Japan. Animals were fed twice a day with a standard marmoset diet (CMS-1M, CLEA Japan) supplemented with fruit, eggs and milk. Water was given ad libitum. The animals were in healthy condition and confirmed to be negative for anti-dengue-virus antibodies before inoculation with dengue virus [24].

Cells

Cell culture was performed as described previously [24]. Vero cells were cultured in minimum essential medium (MEM, Sigma) with 10 % heat-inactivated fetal bovine serum (FBS, GIBCO) and 1 % non-essential amino acid (NEAA, Sigma) at 37 °C in 5 % CO_2. C6/36 cells were cultured in MEM with 10 % FBS and 1 % NEAA at 28 °C in 5 % CO_2.

Virus

DENV type 2 (DENV-2) strain DHF0663 (accession no. AB189122) and strain D2/Hu/Maldives/77/2008NIID (Mal/77/08) were used for inoculation studies. The DENV-2, DHF0663 strain was isolated from a DHF case in Indonesia. The DENV-2 Mal/77/08 strain was isolated from imported DF cases from the Maldives. For all DENV strains, isolated clinical samples were propagated in C6/36 cells and were used within four passages on C6/36 cells. Culture supernatant from infected C6/36 cells was

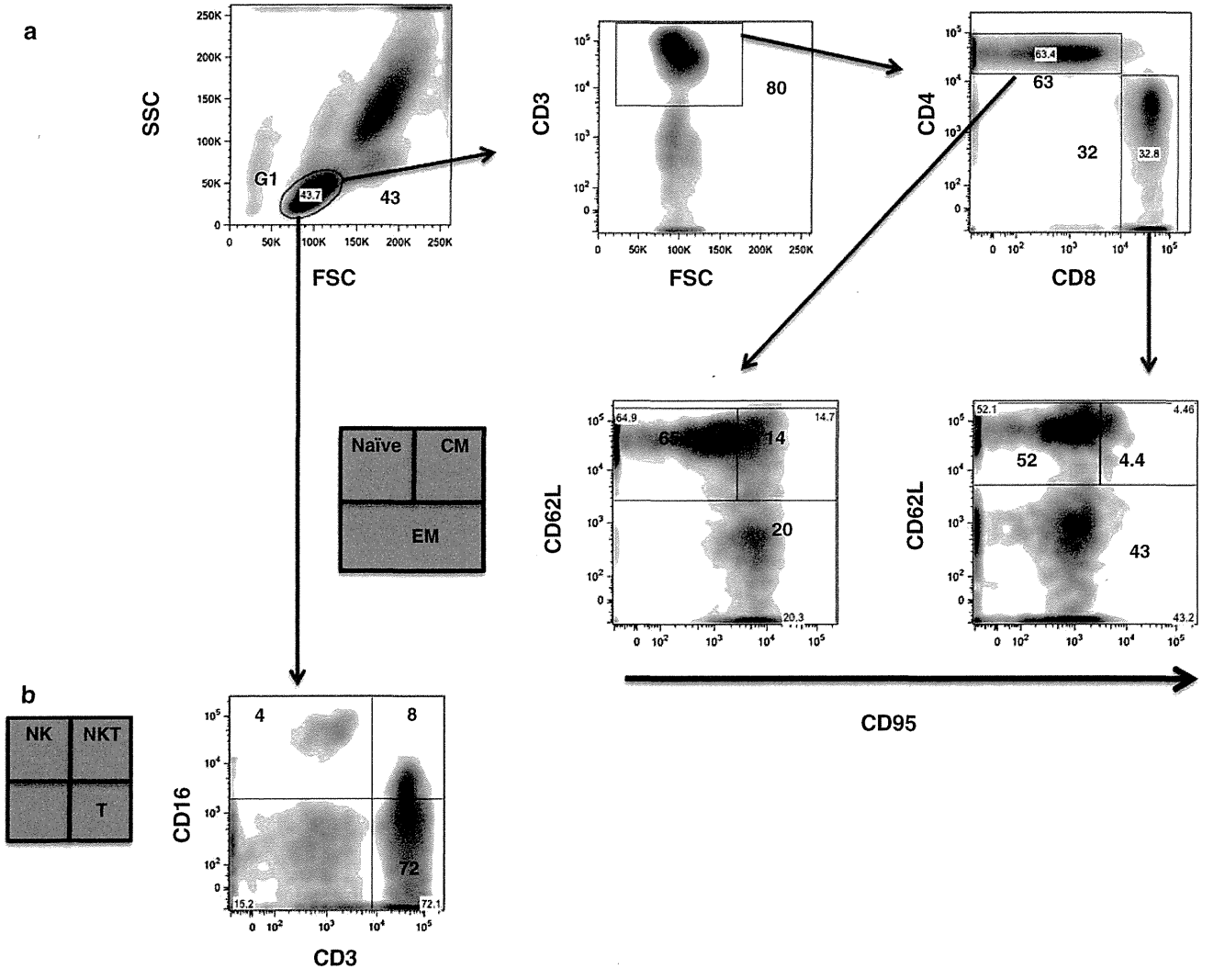

Fig. 1 Flow cytometric analysis of naïve, central/effector memory T cells and NK/NKT cells in marmosets. (**a**) Gating strategy to indentify CD4 and CD8 T, NK and NKT cells. The G1 population was selected and analyzed for CD4 and CD8 T, NK and NKT cells. (**a**) Profiling of naïve, central memory, and effector memory CD4 and CD8 T cells in total CD4 and CD8 T cells. (**b**) Profiling of NK and NKT cells in total lymphocytes. Results shown are representative of three healthy marmosets used in this study

centrifuged at 3,000 rpm for 5 min to remove cell debris and then stored at −80 °C until use.

Infection of the marmosets with DENV

In the challenge experiments, profiling of the key adaptive and innate immune cells in the marmosets after infection with DENV-2 was done. For primary DENV infection, four marmosets were inoculated subcutaneously in the back with either 1.9×10^5 PFU of the DENV-2 Mal/77/08 strain (Cj08-007, Cj07-011) or 1.8×10^4 PFU of the DHF0663 strain (Cj07-006, Cj07-008) [24]. In the case of the DENV re-challenge experiment, two marmosets initially inoculated with 1.8×10^5 PFU of the DHF0663 strain were re-inoculated 33 weeks after the primary challenge with 1.8×10^5 PFU of the same strain (Cj07-007, Cj07-014) [24]. Blood samples were collected on days 0, 1, 3, 7, 14, and 21 after inoculation and were used for virus titration and flow cytometric analysis. Inoculation with DENV and blood drawing were performed under anesthesia with 5 mg/kg of ketamine hydrochloride. Day 0 was defined as the day of virus inoculation. The viral loads in marmosets obtained in a previous study are shown in Supplementary Figure 1 [24].

Flow cytometry

Flow cytometry was performed as described previously [37]. Fifty microliters of whole blood from marmosets was stained with combinations of fluorescence-conjugated monoclonal antibodies; anti-CD3 (SP34-2; Becton Dickinson), anti-CD4 (L200; BD Pharmingen), anti-CD8 (CLB-T8/4H8; Sanquin), anti-CD16 (3G8; BD Pharmingen), anti-CD95 (DX2; BD Pharmingen), and anti-CD62L (145/15; Miltenyi Biotec). Then, erythrocytes were lysed with FACS lysing solution (Becton Dickinson). After washing with a sample buffer containing phosphate-buffered saline (PBS) and 1 % FBS, the labeled cells were resuspended in a fix buffer containing PBS and 1 % formaldehyde. The expression of these markers on the lymphocytes was analyzed using a FACSCanto II flow cytometer (Becton Dickinson). The data analysis was conducted using FlowJo software (Treestar, Inc.). Results are shown as mean ± standard deviation (SD) for the marmosets used in this study.

Table 1 Subpopulation ratios of lymphocytes in marmosets

Subpopulation name	Subpopulation ratios (Mean ± SD: %)
$CD3^+$	75.7 ± 6.4
$CD3^+CD4^+$	65.4 ± 6.8
$CD3^+CD4^+CD62L^+CD95^-$ (CD4 T_N)	65.9 ± 3.7
$CD3^+CD4^+CD62L^+CD95^+$(CD4 T_{CM})	16.4 ± 2.9
$CD3^+CD4^+CD62LCD95^\pm$ (CD4 T_{EM})	19.5 ± 2.5
$CD3^+CD8^+$	29.0 ± 8.0
$CD3^+CD8^+CD62L^+CD95^-$ (CD8 T_N)	66.7 ± 10.2
$CD3^+CD8^+CD62L^+CD95^+$(CD8 T_{CM})	4.7 ± 3.6
$CD3^+CD8^+CD62LCD95^\pm$ (CD8 T_{EM})	28.8 ± 14.8
CD3CD16 + (NK)	4.2 ± 2.6
$CD3^+CD16^+$ (NKT)	5.1 ± 3.4

SD: Standard deviation

Results shown are mean ± SD from 3 healthy marmosets

Results

Naïve central/effector memory T cells and NK/NKT cells in marmosets

Basic information regarding CD4/CD8 naïve and central/effector memory T cells and NK/NKT cells in common marmosets was unavailable. Thus, we examined the immunophenotypes of lymphocyte subsets in the marmosets (Fig. 1). The gating strategy for profiling the CD4 and CD8 T cells in the marmosets by FACS is shown in Fig. 1a. Human T cells are classically divided into three functional subsets based on their cell-surface expression of CD62L and CD95, i.e., $CD62L^+CD95^-$ naive T cells (T_N), $CD62L^+CD95^+$ central memory T cells (T_{CM}), and $CD62L^-CD95^\pm$ effector memory T cells (T_{EM}) [9, 21, 28]. In this study, $CD4^+$ and $CD8^+$ T_N, T_{CM}, and T_{EM} subpopulations were defined as $CD62L^+CD95^-$, $CD62L^+CD95^+$, and $CD62L^-CD95^\pm$, respectively (Fig. 1a and Table 1). The average ratio of $CD3^+$ T lymphocytes in the total lymphocytes of three marmosets was found to be 75.7 ± 6.4 %. The average ratio of $CD4^+$ T cells in the $CD3^+$ subset was 65.4 ± 6.8 %. The average ratios of $CD4^+$ T_N, T_{CM}, and T_{EM} cells were 65.9 ± 3.7 %, 16.4 ± 2.9 %, 19.5 ± 2.5 %, respectively. The average ratio of $CD8^+$ T cells in the $CD3^+$ subset was 29.0 ± 8.0 %. The average ratios of $CD8^+$ T_N, T_{CM}, and T_{EM} cells were 66.7 ± 10.2 %, 4.7 ± 3.6 %, 28.8 ± 14.8 %, respectively.

We recently characterized a $CD16^+$ major NK cell subset in tamarins and compared NK activity in tamarins with or without DENV infection [37, 38]. In terms of NKT cells, NK1.1 (CD161) and CD1d are generally used as markers of NKT cells [32]. However, these anti-human NK1.1 and CD1d antibodies are unlikely to cross-react with the NKT cells of the marmosets. Thus, we defined NKT cells as a population expressing both CD3 and CD16 as reported previously [14, 17]. The NK and NKT cell subsets were determined to be $CD3^-CD16^+$ and $CD3^+CD16^+$ lymphocytes in the marmosets. The average ratios of NK and NKT cell subsets in the lymphocytes were 4.2 ± 2.6 % and 5.1 ± 3.4 %, respectively (Table 1). We observed that the proportions of the major lymphocyte

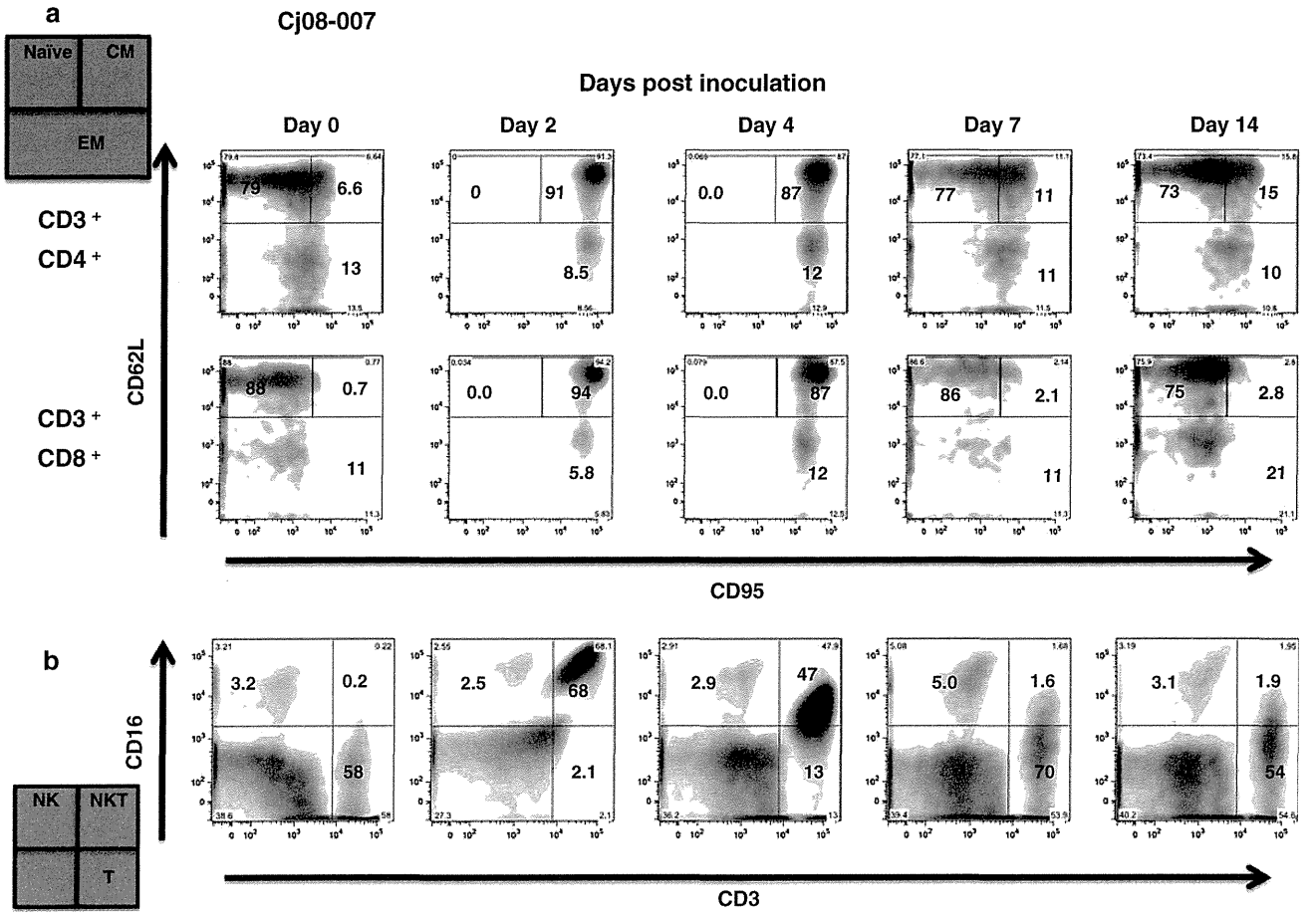

Fig. 2 Profiling of CD4 and CD8 T, NK and NKT cells in marmosets with primary infection with the DENV-2 Mal/77/08 strain. For primary DENV infection, two marmosets were inoculated subcutaneously in the back with 1.9×10^5 PFU of the DENV-2 Mal/77/08 strain. (**a**) Profiling of naïve, central memory, and effector memory CD4 and CD8 T cells in total CD4 and CD8 T cells. (**b**) Profiling of NK and NKT cells in total lymphocytes. (**a-b**) Cj08-007

subsets in the marmosets were similar to those in cynomolgus monkeys and tamarins [37, 38].

Profiling of CD4 and CD8 T, NK and NKT cells in marmosets after primary infection with DENV-2 (Mal/77/08 strain)

We investigated the cellular immune responses against DENV-2 DF strain (Mal/77/08) in marmosets. Dengue vRNA was detected in plasma samples from two marmosets on day 2 postinfection (Supplementary Fig. 1a). For the two marmosets (Cj08-007, Cj07-011), the plasma levels of vRNA reached their peaks at 9.6×10^6 and 7.0×10^6 GE/ml, respectively, on day 4 postinfection. Plasma vRNA was detected in both marmosets on days 2, 4, and 7. We then examined the profiles and frequencies of the CD4 and CD8 T, NK and NKT cells in the infected marmosets (Figs. 2–3 and Table 2). $CD4^+$ T_{CM} cells drastically increased to 88.7 ± 2.8 % from 13 ± 0.4 % between day 0 and day 2 post-inoculation (Table 2). Reciprocally, $CD4^+$ T_N cells decreased to 1.6 ± 3.3 % from 74.1 ± 0.9 % at the same time. $CD4^+$ T_{EM} cells maintained the initial levels throughout the observation period. $CD8^+$ T_{CM} cells increased to 91.9 ± 5.5 % from 2.1 ± 0.8 % between day 0 day 2 post-inoculation, and reciprocally, $CD8^+$ T_N cells decreased to 2.5 ± 4.7 % from 89.9 ± 2.5 % at the same time. In addition, NK cells maintained their initial levels throughout the observation period. However, NKT cells drastically increased to 52.6 ± 17 % from 0.2 ± 0.0 % between day 0 and day 2 post-inoculation. These results suggest that CD4/CD8 T and NKT cells may efficiently respond to the Mal/77/08 strain of DENV.

Profiling of CD4 and CD8 T, NK and NKT cells in the marmosets after primary infection with DENV-2 (DHF0663 strain)

Next, we investigated cellular immune responses against another DENV-2 DHF strain (DHF0663) in marmosets.

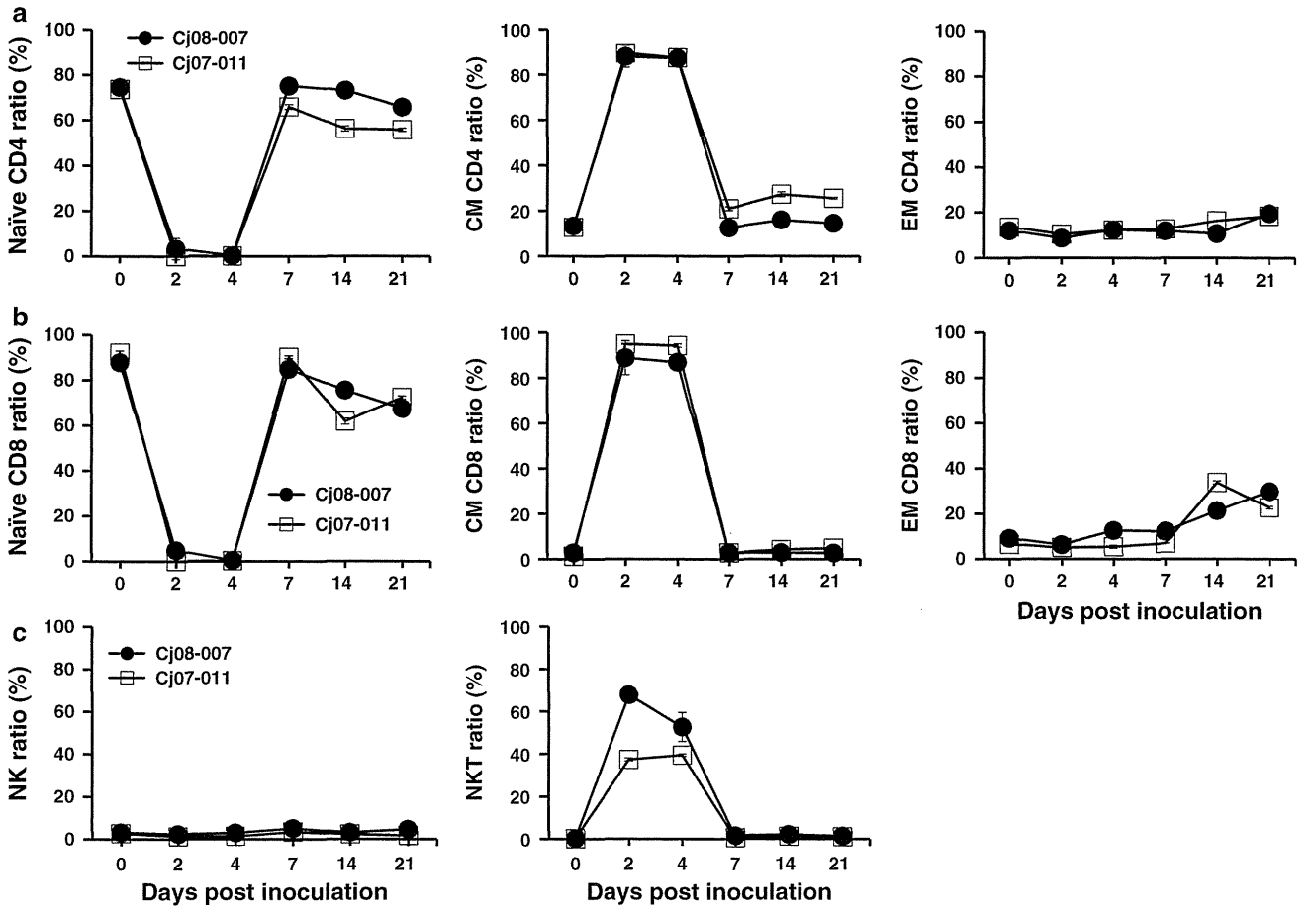

Fig. 3 Frequency of CD4 and CD8 T, NK and NKT cells in marmosets with primary infection with the DENV-2 Mal/77/08 strain. For primary DENV infection, two marmosets were inoculated subcutaneously in the back with 1.9×10^5 PFU of the DENV-2 Mal/77/08 strain. (**a**) Ratios of naïve, central memory, and effector memory CD4 T cells in total CD4 T cells. (**b**) Ratios of naïve, central memory, and effector memory CD8 T cells in total CD8 T cells. (**c**) Ratios of NK and NKT cells in total lymphocytes. (**a-c**) Cj08-007, Cj07-011

Table 2 Subpopulation ratios of lymphocytes in marmosets during primary DENV infection (Mal/77/08)

Subpopulation name		Subpopulation ratio (Mean ± SD: %)					
		Days after inoculation					
		Day 0	Day 2	Day 4	Day 7	Day 14	Day 21
$CD3^+CD4^+CD62L^+CD95''$	($CD4\ T_N$)	74.1 ± 0.9	1.6 ± 3.3	0.2 ± 0.3	70.5 ± 5.5	64.8 ± 9.7	60.8 ± 5.9
$CD3^+CD4^+CD62L^+CD95^+$	($CD4\ T_{CM}$)	13 ± 0.4	88.7 ± 2.8	87.4 ± 0.2	16.8 ± 5.0	21.6 ± 6.5	20 ± 6.4
$CD3^+CD4^+CD62LCD95^\pm$	($CD4\ T_{EN}$)	12.8 ± 0.9	9.5 ± 1.0	12.3 ± 0.4	12.3 ± 0.5	134 ± 3.2	189 ± 1.4
$CD3^+CD8^+CD62L^+CD95^-$	($CD8\ T_N$)	89.9 ± 2.5	2.5 ± 4.7	0.3 ± 0.3	87.5 ± 3.3	68.7 ± 79	69.8 ± 3.1
$CD3^+CD8^+CD62L^+CD95^+$	($CD8\ T_{CM}$)	2.1 ± 0.8	91.9 ± 5.5	90.6 ± 4.2	2.8 ± 0.5	3.5 ± 08	3.8 ± 1.2
$CD3^+CD8^+CD62LCD95^\pm$	($CD8\ T_{EN}$)	7.8 ± 1.6	5.6 ± 0.8	9.0 ± 4.1	9.5 ± 3.1	27.6 ± 72	26.3 ± 4.3
$CD3^-CD16+$	(NK)	2.9 ± 0.2	1.8 ± 0.6	2.2 ± 0.9	4.2 ± 0.9	2.8 ± 04	3.2 ± 1.7
$CD3^+CD16^+$	(NKT)	0.2 ± 0.0	52.6 ± 17	46.1 ± 8.5	1.1 ± 05	1.7 ± 05	1.2 ± 0.2

SD: Standard deviation

Results shown are mean ± SD from two marmosets as shown in Figure 3

Dengue vRNA was detected in plasma samples from the marmosets on day 2 post-infection ([24], Supplementary Fig. 1b). For the two marmosets (Cj07-006, Cj07-008), the plasma vRNA levels were found to be 3.4×10^5 and 3.8×10^5 GE/ml on day 2 and 2.0×10^6 and 9.4×10^5 GE/ml, respectively, at the peak on day 4 post-infection and became undetectable by day 14. Thus, we examined the profiles and frequencies of the $CD4^+$ and $CD8^+$ T, NK and NKT cells in these DENV-infected marmosets (Fig. 4–5 and Table 3). It was found that on day 7 post-inoculation, $CD4^+$ and $CD8^+$ T_N cells decreased, and in contrast, the T_{CM} populations increased in both marmosets; however, the changes in proportion were much less pronounced than in the case of the marmosets infected with the DF strain. We observed no consistent tendency in the kinetics of $CD4^+$ and $CD8^+$ T_{EM} cells nor in NK and NKT cells. These results suggest that the strength of T cell responses may be dependent on the strain of DENV.

Profiling of CD4 and CD8 T, NK and NKT cells in marmosets re-challenged with a DENV-2 strain

In order to examine the cellular immune responses against re-challenge with a DENV-2 DHF strain in the marmoset model, marmosets were infected twice with the same DENV-2 strain (DHF0663) with an interval of 33 weeks after the primary infection. The results showed that vRNA and NS1 antigens were not detected in plasma and that the neutralizing antibody titer was obviously increased after the secondary infection. The data indicated that the primary infection induced protective immunity, including a neutralizing antibody response to re-challenge with the same DENV strain ([24]; Supplementary Fig. 1c). We also investigated the profiles of the CD4 and CD8 T, NK and NKT cells in the marmosets (Cj07-007, Cj07-014) that were re-challenged with the same DENV-2 strain (DHF0663) (Figs. 6–7). $CD4^+$ T_{CM} cells drastically increased on day 14 post-inoculation. On the other hand,

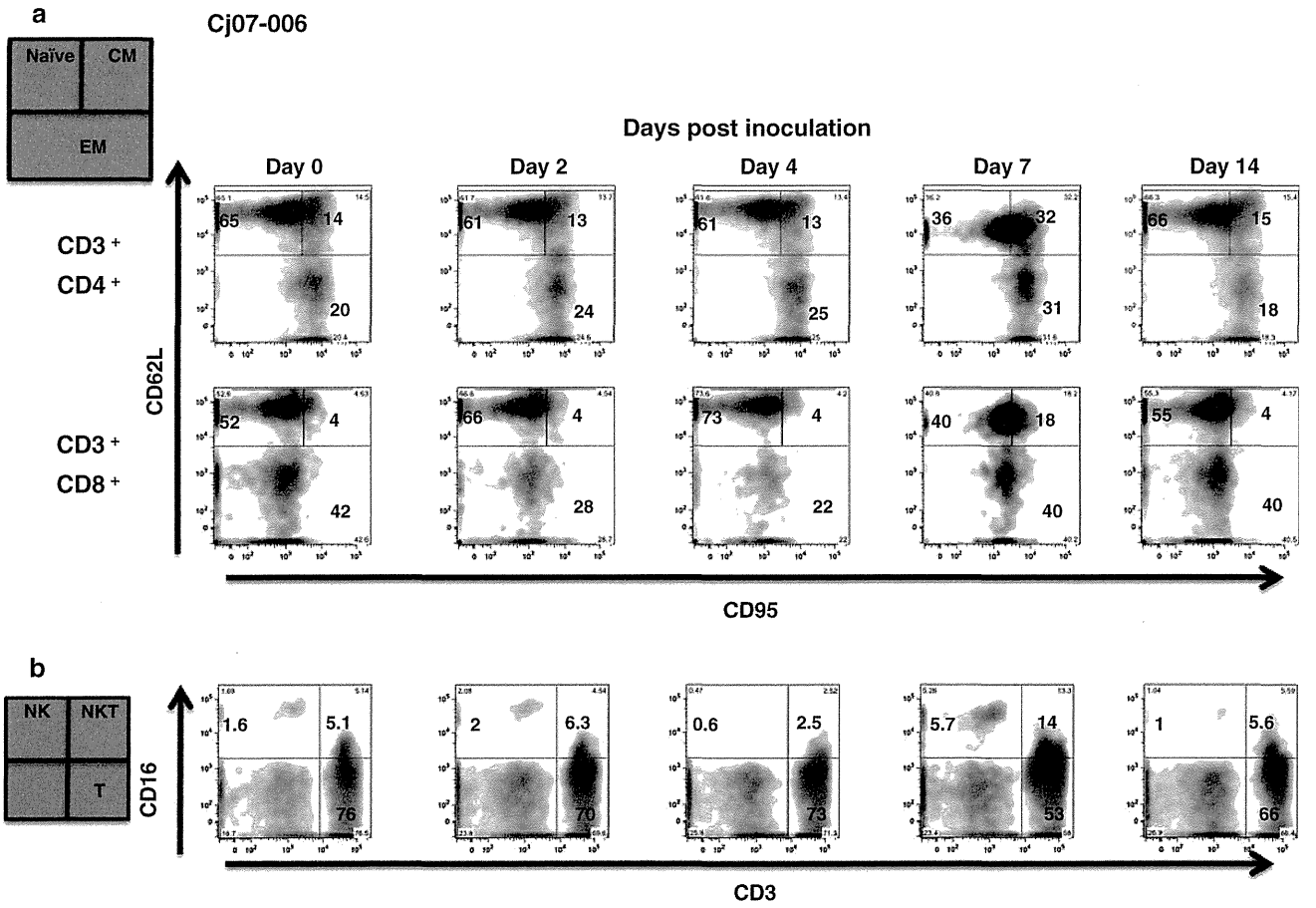

Fig. 4 Profiling of CD4 and CD8 T, NK and NKT cells in marmosets with primary infection with the DENV-2 DHF0663 strain. For primary DENV infection, two marmosets were inoculated subcutaneously in the back with 1.8×10^4 PFU of the DENV-2 DHF0663 strain. (**a**) Profiling of naïve, central memory, and effector memory CD4 and CD8 T cells in total CD4 and CD8 T cells. (**b**) Profiling of NK and NKT cells in total lymphocytes. (**a-b**) Cj07-006

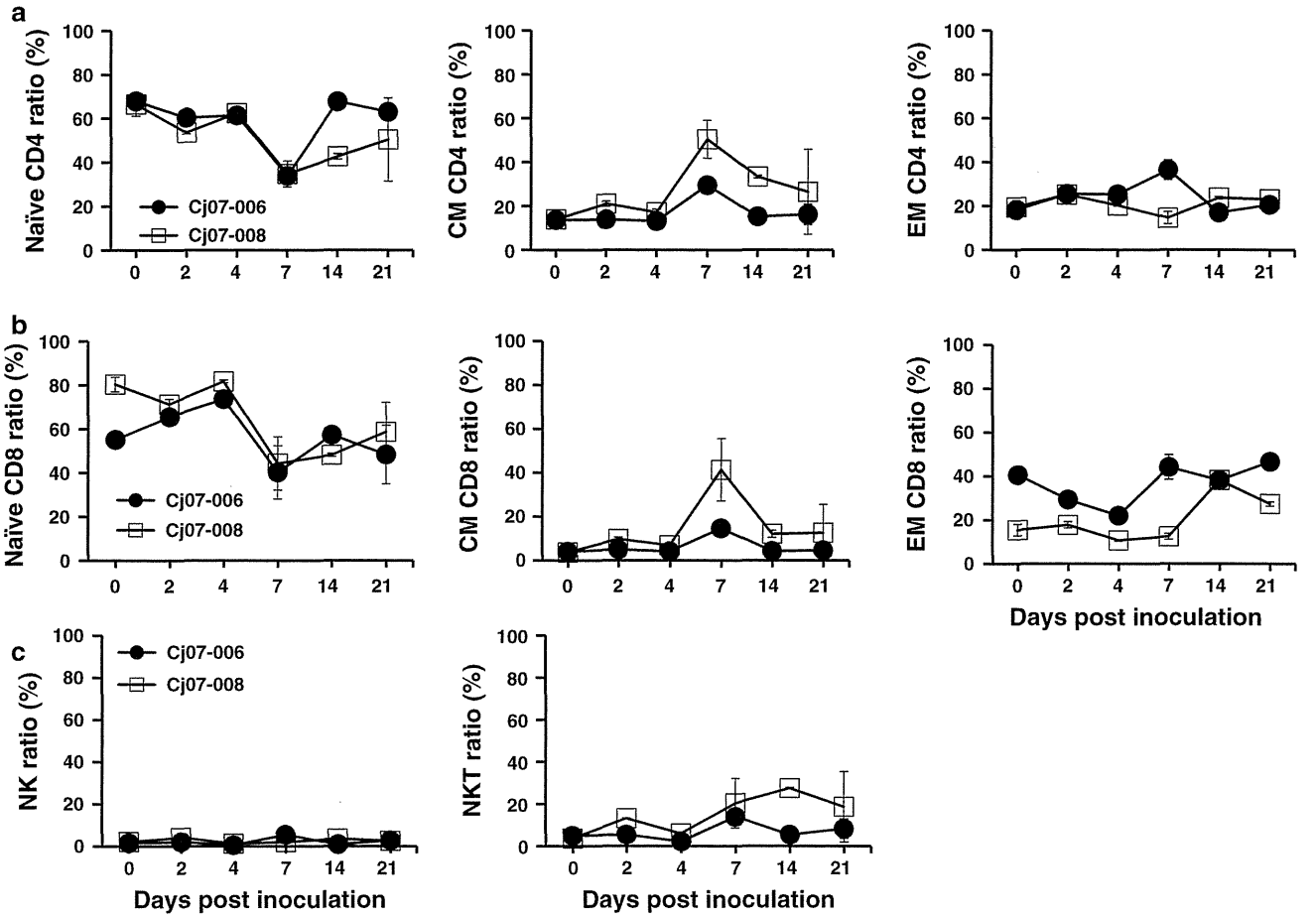

Fig. 5 Frequency of CD4 and CD8 T, NK and NKT cells in marmosets with primary infection with the DENV-2 DHF0663 strain. For primary DENV infection, two marmosets were inoculated subcutaneously in the back with 1.8×10^4 PFU of the DENV-2 DHF0663 strain. (**a**) Ratios of naïve, central memory, and effector memory CD4 T cells in total CD4 T cells. (**b**) Ratios of naïve, central memory, and effector memory CD8 T cells in total CD8 T cells. (**c**) Ratios of NK and NKT cells in total lymphocytes. (**a-c**) Cj07-006, Cj07-008

Table 3 Subpopulation ratios of lymphocytes in marmosets during primary DENV infection (DHF0663)

Subpopulation name		Subpopulation ratios (Mean ± SD: %)					
		Days after inoculation					
		Day 0	Day 2	Day 4	Day 7	Day1 4	Day 21
$CD3^+CD4^+CD62L^+CD95^-$	($CD4\ T_N$)	67.3 ± 3.6	57.0 ± 4.0	61.9 ± 0.9	34.4 ± 3.6	55.2 ± 14	56.7 ± 13
$CD3^+CD4^+CD62L^+CD95^+$	($CD4T_{CM}$)	13.9 ± 1.3	17.5 ± 4.1	15.2 ± 2.5	40.0 ± 13	33.8 ± 10	21.3 ± 12
$CD3^+CD8^+CD62L^-CD95^\pm$	($CD4\ T_{EM}$)	18.8 ± 2.2	25.3 ± 0.9	22.8 ± 2.9	25.6 ± 13	20.3 ± 4.0	21.8 ± 1.5
$CD3^+CD8^+CD62L^+CD95^-$	($CDS\ T_N$)	67.8 ± 14	68.4 ± 3.7	77.7 ± 4.6	42.2 ± 7.4	52.7 ± 5.5	53.5 ± 9.8
$CD3^+CD8^+CD62L^+CD95^-$	($CDS\ T_{CM}$)	3.9 ± 0.6	7.4 ± 2.8	5.5 ± 1.6	28 ± 17	8.1 ± 4.6	8.6 ± 8.9
$CD3^+CD8^+CD62L^-CD95^\pm$	($CDS\ T_{EM}$)	28 ± 14	23.5 ± 6.7	16.4 ± 6.5	28.3 ± 18	38.2 ± 1.9	37.0 ± 11
$CD3^-CD16^+$	(NK)	4.7 ± 1.0	4.2 ± 1.9	2.0 ± 1.1	6.3 ± 2.3	5.1 ± 2.2	7.3 ± 1.2
$CD3^+CD16^+$	(NKT)	7.8 ± 1.0	9.3 ± 4.5	5.9 ± 2.6	22.6 ± 8.4	20.6 ± 10	17.3 ± 10

SD: Standard deviation

Results shown are mean ± SD from 2 marmosets as shown in Figure 5

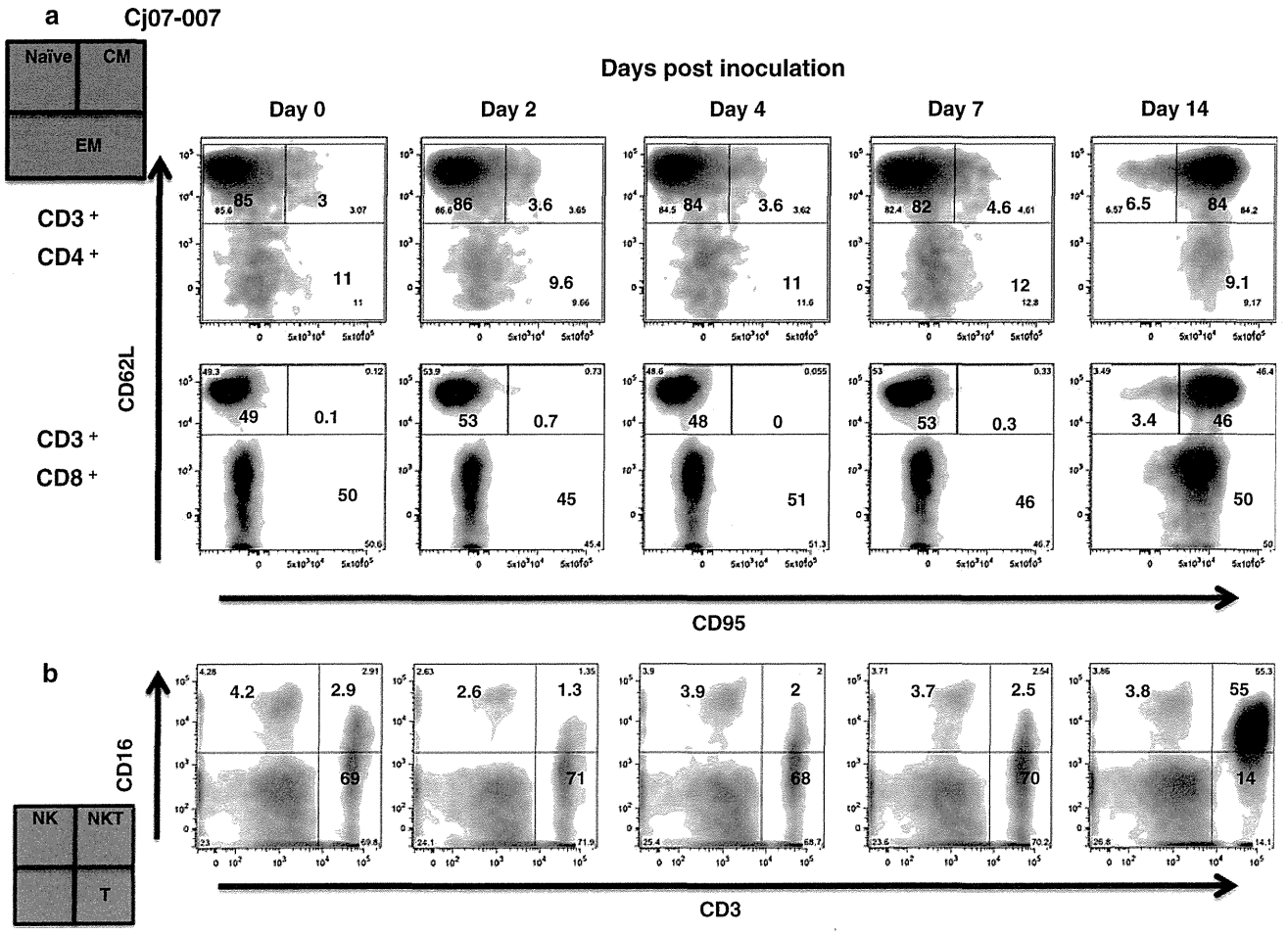

Fig. 6 Profiling of CD4 and CD8 T, NK and NKT cells in marmosets after re-challenge with the DENV-2 DHF0663 strain. Two marmosets that were initially inoculated with 1.8×10^5 PFU of the DHF0663 strain were re-inoculated 33 weeks after the primary challenge with 1.8×10^5 PFU of the same strain. (**a**) Profiling of naïve, central memory, and effector memory CD4 and CD8 T cells in total CD4 and CD8 T cells. (**b**) Profiling of NK and NKT cells in total lymphocytes. (**a-b**) Cj07-007

$CD4^+$ T_N cells decreased strongly at the same time. $CD4^+$ T_{EM} cells maintained their initial levels through the observation period. Similarly, $CD8^+$ T_{CM} and NKT cells clearly increased on day 14 post-inoculation. Importantly, these T cell responses were induced one week after the obvious induction of the neutralizing antibody in the marmosets [24]. These results suggest that the neutralizing antibody may play a critical role in the complete inhibition of the secondary DENV infection.

Discussion

In this study, we demonstrated the dynamics of the central/effector memory T cells and NK/NKT subsets against DENV infection in our marmoset model. First, we characterized the central/effector memory T and NK/NKT subsets in marmosets (Fig. 1). Second, we found that CD4/CD8 central memory T cells and NKT cells had significant responses in the primary DENV infection, and the levels appeared to be dependent on the strain of the virus employed for challenge experiments (Figs. 2–5). Finally, we found delayed responses of CD4/CD8 central memory T cells in the monkeys re-challenged with the same DENV DHF strain, despite the complete inhibition of DENV replication (Figs. 6–7).

The present study shed light on the dynamics of cellular and humoral immune responses against DENV *in vivo* in the marmoset model. Our results showed that cellular immune responses were induced earlier than antibody responses in the primary infection. Thus, our results suggest the possibility that cellular immunity may contribute, at least in part, to the control of primary DENV infection. On the other hand, in the presence of neutralizing antibodies in the re-challenged monkeys [24], delayed (on day 14 after the re-challenge) responses of CD4/CD8 central memory T cells were observed despite the complete inhibition of DENV replication. These results indicate that

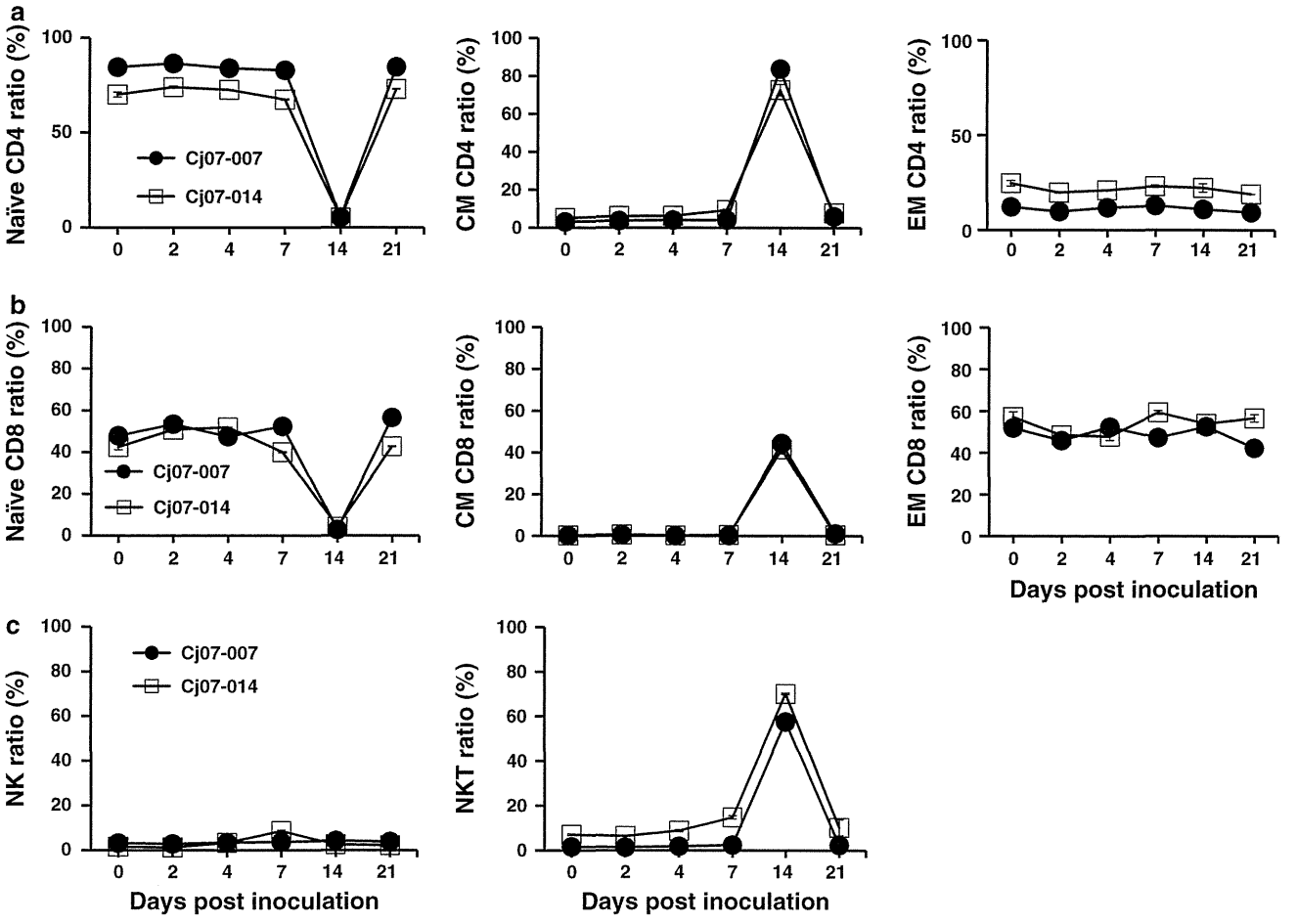

Fig. 7 Frequency of CD4 and CD8 T, NK and NKT cells in marmosets after re-challenge with the DENV-2 DHF0663 strain. Two marmosets initially inoculated with 1.8×10^5 PFU of the DHF0663 strain were re-inoculated 33 weeks after the primary challenge with 1.8×10^5 PFU of the same strain. (**a**) Ratios of naïve, central memory, and effector memory CD4 T cells in total CD4 T cells. (**b**) Ratios of naïve, central memory, and effector memory CD8 T cells in total CD8 T cells. (**c**) Ratios of NK and NKT cells in total lymphocytes. (**a-c**) Cj07-007, Cj07-014

cellular immunity is unlikely to play a major role in the control of DENV re-infection. Alternatively, it is still possible that components of cellular immunity, such as memory T cells, could partially play a helper role for the enhanced induction of neutralizing antibodies even without an apparent increase in the proportion of T_{CM}, resulting in efficient prevention of DENV replication.

It is possible that the DENV strains used in this study influence the strength of cellular immune responses. The differences in cellular immune responses between the monkeys infected with the DF and DHF strains are probably not caused by individual differences in the marmosets, because the FACS results were consistent with each pair of marmosets. It was shown previously that there was a reduction in CD3, CD4, and CD8 cells in DHF and that lower levels of CD3, CD4, and CD8 cells discriminated DHF from DF patients during the febrile stage of illness [5]. There was a significant increase in an early activation marker on $CD8^+$ T cells in children with DHF compared with DF during the febrile period of illness [8]. Another group reported that levels of peripheral blood mononuclear cell apoptosis were higher in children developing DHF [23]. Moreover, cDNA array and ELISA screening demonstrated that IFN-inducible genes, IFN-induced genes and IFN production were strongly up-regulated in DF patients when compared to DHF patients, suggesting a significant role of the IFN system during infection with DF strains when compared to DHF strains [34]. Thus, it is reasonable to assume that DHF strains might have the ability to negatively regulate T cell responses. A recent report demonstrating that the sequence of a DHF strain differed from that of a DF strain at six unique amino acid residues located in the membrane, envelope and non-structural genes [33], which supports our notion.

Alternatively, another possibility is that the strength of T cell responses might depend on the viral load. In fact, in

our results, the stronger T cell responses in monkeys infected with the DF strain were paralleled by higher viral loads, which was in contrast to the result obtained with DHF-strain-infected animals with lower viral loads. Of note, the tenfold higher challenge dose of the DF strain used in this study (1.9×10^5 PFU) compared to the DHF strain (1.8×10^4 PFU) could have simply led to tenfold higher peak viral RNA levels in monkeys infected with the DF strain. In either case, the relationship between the strength of the antiviral immune response and the viral strain remains to be elucidated. Further *in vivo* characterization of the antiviral immunity and the viral replication kinetics induced by infection with various DENV strains isolated from DF and DHF patients will help to understand the mechanism of differential disease progression in the course of DENV infection.

We observed that dengue vRNA was not detected in plasma samples from marmosets re-infected with the same DENV-2 DHF strain 33 weeks after the primary infection. This result suggests that memory B cells induced in the primary DENV infection were predominantly activated to produce neutralizing antibodies against the same DHF strain in the secondary infection in the absence of apparent cellular immune responses. A previous report showed that DENV infection induces a high-titered neutralizing antibody that can provide long-term immunity to the homologous DENV serotype [22], which is consistent with our results. By contrast, the role of cellular immune responses in the control of DENV infection remains to be elucidated. Our results in this study may suggest that cellular immune responses and neutralizing antibodies acted cooperatively to control primary DENV infection. In DENV-infected patients, it may be difficult to distinguish whether each case is primary or secondary DENV infection and also to serially collect blood samples for immunological study in the course of the infection, which is likely to be the reason for the discrepancy regarding the importance of cellular immunity in DENV infection. From this point of view, our marmoset model of DENV infection will further provide important information regarding the role of cellular immune responses in DENV infection.

Acknowledgments We would like to give special thanks to members of The Corporation for Production and Research of Laboratory Primates for technical assistance. We also appreciate Ms. Tomoko Ikoma and Mizuho Fujita for technical assistance. This work was supported by grants from the Ministry of Health, Labor and Welfare of Japan, and by the Environment Research and Technology Development Fund (D-1007) from the Ministry of the Environment of Japan.

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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RESEARCH PAPER

Human Vaccines & Immunotherapeutics 9:2, 283–290; February 2013; © 2012 Landes Bioscience

TLR9 adjuvants enhance immunogenicity and protective efficacy of the SE36/AHG malaria vaccine in nonhuman primate models

Takahiro Tougan,[1] Taiki Aoshi,[2,3] Cevayir Coban,[4] Yuko Katakai,[5] Chieko Kai,[6] Yasuhiro Yasutomi,[7] Ken J. Ishii[2,3,†,*] and Toshihiro Horii[1,†,*]

[1]Department of Molecular Protozoology; Research Institute for Microbial Diseases; Osaka University at Suita; Osaka, Japan; [2]Laboratory of Adjuvant Innovation; National Institute of Biomedical Innovation (NIBIO) at Ibaraki; Osaka, Japan; [3]Laboratory of Vaccine Science; Immunology Frontier Research Center (IFReC); Osaka University at Suita; Osaka, Japan; [4]Laboratory of Malaria Immunology; Immunology Frontier Research Center (IFReC); Osaka University at Suita; Osaka, Japan; [5]The Corporation for Production and Research of Laboratory Primates at Tsukuba; Ibaragi, Japan; [6]Laboratory Animal Research Center; The Institute of Medical Science; The University of Tokyo at Minato-ku; Tokyo, Japan; [7]Tsukuba Primate Research Center; National Institute of Biomedical Innovation at Tsukuba; Ibaragi, Japan

[†]These authors contributed equally to this work.

Keywords: SE36, malaria vaccine, *Plasmodium falciparum*, TLR9 ligand adjuvant, synthetic hemozoin, CpG ODN, nonhuman primate model

Abbreviations: SERA, serine repeat antigen; AHG, aluminum hydroxide gel; ODN, oligodeoxyribonucleotides; sHZ, synthetic hemozoin; GMP, good manufacturing practice

The SE36 antigen, derived from serine repeat antigen 5 (SERA5) of *Plasmodium falciparum*, is a promising blood stage malaria vaccine candidate. Ongoing clinical trials suggest the efficacy of the SE36 vaccine could be increased by the incorporation of more effective adjuvants into the vaccine formulation. In this study, we assessed the safety, immunogenicity and protective efficacy of SE36/AHG formulated with TLR9 ligand adjuvants K3 CpG oligodeoxyribonucleotides (CpG ODNs) (K3 ODN), D3 ODN or synthetic hemozoin, in two non-human primate models. SE36/AHG with or without each adjuvant was administrated to cynomolgus monkeys. A combination of TLR9 ligand adjuvant with SE36/AHG induced higher humoral and cellular immune response compared with SE36/AHG alone. Administration of a crude extract of *P. falciparum* parasite resulted in the induction of more SE36-specific IgG antibodies in monkeys vaccinated with a combination of SE36/AHG and adjuvant, as opposed to vaccination with SE36/AHG alone. The most effective TLR9 ligand, K3 ODN, was chosen for further vaccine trials in squirrel monkeys, in combination with SE36/AHG. All monkeys immunized with the combined SE36/AHG and K3 ODN formulation effectively suppressed parasitemia and symptoms of malaria following challenge infections. Furthermore, no serious adverse events were observed. Our results show that the novel vaccine formulation of K3 ODN with SE36/AHG demonstrates safety, potent immunogenicity and efficacy in nonhuman primates, and this vaccine formulation may form the basis of a more effective malaria vaccine.

Introduction

The fight against malaria is on-going across several fronts. Although progress has been made in combating malaria, there is no question that a key tool against this parasite would be an effective vaccine. As the malaria vaccine portfolio proceeds through development, an increasing number of clinical trials have confirmed the critical importance of presenting the most appropriate antigen/adjuvant in a formulation that will likely elicit the desired immune response.[1]

This was illustrated in trials of RTS,S-based vaccines where only 2/7 individuals were protected with an in oil-in water emulsion, whereas 6/7 individuals were fully protected when the antigen was formulated in this emulsion and supplemented with the immune stimulants monophosphoryl lipid A and QS21.[2] In studies using long synthetic peptides from the conserved C-terminal region of merozoite surface protein (MSP)-3 formulated with Alhydrogel, antibody titers were sustained for over a year. A biological activity of the antibodies against *P. falciparum* as determined in vitro by antibody-dependent cellular inhibition (ADCI) and in vivo by passive transfer in *P. falciparum*-infected SCID mice. In vitro, the antibodies induced an inhibition of the *P. falciparum* erythrocytic growth in a monocyte-dependent manner, which was in most instances as high as or greater than that induced by natural antibodies from immune African adults. In vivo transfer of the volunteers' sera into *P. falciparum*–infected

*Correspondence to: Ken J. Ishii and Toshihiro Horii; Email: kenishii@biken.osaka-u.ac.jp and horii@biken.osaka-u.ac.jp
Submitted: 09/03/12; Revised: 11/07/12; Accepted: 11/18/12
http://dx.doi.org/10.4161/hv.22950

humanized SCID mice profoundly reduced or abrogated parasitemia. These inhibitory effects were related to the antibody reactivity with the parasite native protein, which was seen in 60% of the volunteers, and remained in samples taken one year postimmunization.[3]

The blood-stage antigen SERA5 (for a review see ref. 4) is a promising blood-stage vaccine candidate against *P. falciparum*. SERA5 is largely produced during the late trophozoite and schizont stages.[5-7] Recombinant SE47' antigen, based on the SERA5 molecule, conferred protective immunity against parasite challenge in both *Aotus* and squirrel monkeys.[8-11] Mouse and rat antibodies against the SE47', likewise, inhibited parasite growth in vitro.[12-14] However, SE47' is highly hydrophobic, making large-scale manufacturing under good manufacturing practice (GMP) conditions a major challenge. Therefore, we constructed a new recombinant antigen, SE36, lacking the serine repeats. SE36 absorbed to the adjuvant aluminum hydroxide gel (SE36/AHG) was prepared under GMP conditions. SE36/AHG was highly immunogenic and anti-SE36 IgG titers lasted more than 1 y in chimpanzees.[15] Squirrel monkeys vaccinated with SE36/AHG were protected against high parasitemia, and serum anti-SE36 IgG titers were boosted after malaria parasite challenge. A human phase 1a clinical trial in Japan demonstrated that SE36/AHG (100 μg/1,000 μg) was safe, well-tolerated and immunogenic.[15] However, the mean titer of induced anti-SE36 antibody in the phase 1a trial was lower than that in African high responders.

Synthetic oligodeoxyribonucleotides (ODNs) containing immunostimulatory unmethylated cytosine-guanosine dinucleotides (CpG motifs) are potentially useful adjuvants and have been evaluated for veterinary and human vaccines.[16] These so-called CpG ODNs are categorized into two major classes, K- and D-type. K-type ODNs trigger the maturation of dendritic cells and stimulate the production of IgM and interleukin (IL)-6.[17,18] The D-type ODNs trigger antigen-presenting cell (APC) maturation and preferentially induce interferon (IFN)-α and -γ secretion.[18,19] It has been reported that K3 ODN effectively induces IL-6 production and cell proliferation, and that D35 ODN effectively secretes IFN-α in rhesus macaques.[20]

Another TLR9 ligand, synthetic hemozoin (sHZ, also known as β-hematin), is also a potent adjuvant for malarial antigens.[21] Hemozoin, a malaria pigment, is a detoxified product of heme molecules found in food vacuoles of the malaria parasites.[22,23] In previous studies, it was shown that purified HZ activates macrophages, thereby producing pro-inflammatory cytokines, chemokines and nitric oxide. HZ has also been shown to enhance human myeloid dendritic cell maturation.[21,24] Furthermore, the adjuvant function of sHZ was validated in a canine antiallergenic vaccine model.[25] Thus, HZ can influence adaptive immune responses to malaria infection and may have therapeutic value in vaccine adjuvant development.

We recently determined that a formulation of K3 and D35 ODNs, or sHZ with SE36/AHG was effective for the induction of anti-SE36 IgG in a rodent malaria model (Tougan et al., unpublished data). The purpose of this study was to increase the levels of induced antibody using a vaccine formulation containing TLR9 ligands as adjuvants. Here, we report the safety, immunogenicity and protective efficacy of the SE36/AHG formulation containing either K3 ODN, D35 ODN or sHZ as an adjuvant in non-human primate models.

Results

Adjuvant efficacy of TLR9 ligands to SE36/AHG. The adjuvant efficacy of K3 and D35 ODNs, and sHZ with SE36/AHG was examined. Twelve cynomolgus monkeys were randomly assigned to four groups. SE36/AHG, with or without each adjuvant, was administered four times and SE36-specific IgG titer was measured. Two weeks after the second immunization (Day 36), mean anti-SE36 antibody titers were 54.5, 432.9 ($p < 0.05$), 68.8 and 270.1 in the SE36/AHG, SE36/AHG with K3 ODN, SE36/AHG with D35 ODN and SE36/AHG with sHZ groups, respectively (**Fig. 1A and B**). Two weeks after the third immunization (Day 112), mean antibody titers were 182.2, 2258.9 ($p < 0.05$), 704.3 and 1276.1, respectively (**Fig. 1A and B**). Two weeks after the fourth immunization (Day 379), mean antibody titers were 237.8, 1960.8 ($p < 0.05$), 548.2 and 791.3 in the respective groups (**Fig. 1A and B**). At the three time points post-administration (**Fig. 1A and B**), the formulations including K3 ODN and sHZ elicited significantly higher anti-SE36 antibody titers. In particular, K3 ODN remarkably enhanced the antibody response after each administration. Furthermore, titers in individual monkeys from each group were compared immediately before each immunization to observe sustainability of the antibody titers. After the second immunization, mean antibody titers were 13.9, 176.0, 17.3 and 43.0 in the SE36/AHG, SE36/AHG with K3 ODN, SE36/AHG with D35 ODN, and SE36/AHG with sHZ groups, respectively (**Fig. 1A and B**). After the third immunization, mean antibody titers were 17.6, 157.7, 26.6 and 21.3, respectively (**Fig. 1A and B**). After the fourth immunization, mean antibody titers were 24.6, 164.5, 94.1 and 24.9 in the respective groups (**Fig. 1A and B**). These results indicate that the formulation with K3 ODN, but not sHZ, was able to sustain antibody titers although both were able to elicit statistically significant titers at each immunization time point.

Overall, the formulation of K3 ODN with SE36/AHG most effectively induced and maintained SE36-specific IgG titers. Additionally, there was no statistically significant difference between titers after the third or fourth immunization, with means of 1960.8 and 2258.9, respectively. This result suggests that an initial immunization with two boosters should be sufficient to confer the maximum levels of antibody titer (**Fig. 1A and B**).

Cytokine responses to SE36 stimulation. To examine the involvement of SE36-specific helper T cell responses, we measured cytokine secretion from peripheral blood mononuclear cells (PBMCs) 2 weeks after the second and fourth immunizations. IFN-γ was used as a marker of the Th1 response, IL-5 and IL-13 as markers of the Th2 response, and IL-17 as a marker of the Th17 response [Day 36 (i) and Day 379 (v) in **Fig. 1**]. On Day 36, IFN-γ was significantly induced in the SE36/AHG with K3 ODN group. IL-5 and IL-13 were significantly induced in each group where K3 ODN or sHZ was employed. These results suggest that a formulation containing K3 ODN promotes both Th1 and Th2 responses.

It appears that sHZ promotes the Th2 response only (**Fig. 2A**). On Day 379, IFN-γ, IL-5 and IL-13 were induced in groups containing K3 ODN in the formulation, along with the SE36/AHG and sHZ groups. These results indicate that four immunizations promote both Th1 and Th2 responses. IL-17 was induced in all four groups on Day 36, with cytokines significantly induced in SE36/AHG and sHZ groups, but only slightly induced in groups where K3 ODN and D35 ODN was included in the formulation (**Fig. 2B**). Of interest to us, the production of all cytokines was not prominent in the SE36/AHG with D35 ODN group at both time points despite the clear antibody titer induction in this group.

Immunostimulation using crude extracts of *P. falciparum*. To examine whether SE36-specific IgGs can be boosted by natural SERA5 antigen exposure after natural infection, we injected a crude extract of *P. falciparum* strain 3D7 into cynomolgus monkeys to mimic the complex mixture of *Plasmodium* antigens encountered during natural infection. The crude extract was administered at Day 583 (**Fig. 1**) and SE36-specific IgG titers were measured at 0 (Day 583), 1 and 2 weeks after injection. Although there was no statistical difference between week 1 and 2, in all groups, SE36-specific IgG titers were enhanced after injection (**Fig. 3**). We, thus, speculate that SE36-specific IgG antibodies could be boosted by malaria infection.

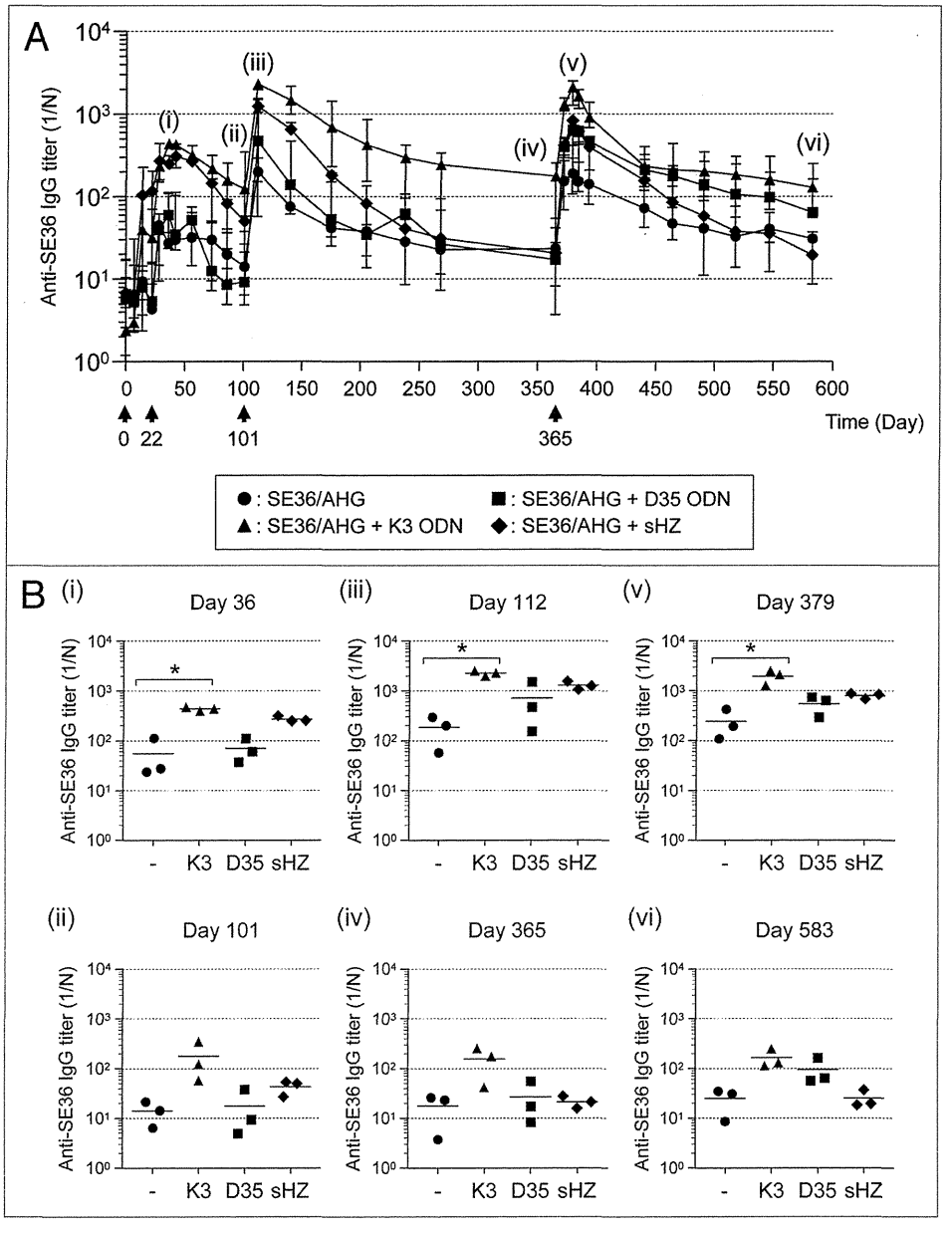

Figure 1. Formulation with K3 ODN effectively enhances SE36-specific IgG titers. (**A**) Time-course of SE36-specific IgG antibody titer. Cynomolgus monkeys were administered the same dose on days 0, 22, 101 and 365 (arrows). Sera were collected on days 0, 7, 14, 22, 28, 36, 42, 56, 73, 86, 101, 112, 140, 175, 205, 238, 268, 365, 372, 379, 384, 393, 440, 464, 491, 518, 547 and 583. Closed circles, triangles, squares and diamonds show the median titers (n = 3/group) of SE36/AHG, SE36/AHG with K3 ODN, SE36/AHG with D35 ODN and SE36/AHG with sHZ, respectively. Ranges are shown by bars. (**B**) Titers for individual monkeys subjected to different treatments on days 36 (i), 101 (ii), 112 (iii), 365 (iv), 379 (v) and 583 (vi) are compared. Statistical analysis for four groups was performed using non-parametric ANOVA (Kruskal-Wallis) with Dunn's post-hoc test; * indicates significant difference, $p < 0.05$.

Vaccine trial in squirrel monkeys. To examine whether the formulation containing K3 ODN provided protective immunity against *P. falciparum* or not, a challenge experiment using squirrel monkeys was performed. At nine weeks after the first administration of the vaccine, blood-stage malaria challenge was done by intravenous injection of 5×10^8 infected red blood cells. It is worth mentioning that the formulation containing K3 ODN did not result in significantly higher antibody titers, which differed from the results we observed in cynomolgus monkeys (**Fig. 4A**).

All three monkeys immunized with SE36/AHG combined with K3 ODN, and one monkey immunized with SE36/AHG alone developed less than 30% parasitemia (maximum parasitemia: 22.6%, 26.8% and 13.3% in the SE36/AHG with K3 ODN group; and 22.0% in the SE36/AHG group). One monkey immunized with SE36/AHG, and two monkeys immunized with AHG combined with K3 ODN experienced fulminant infections of 40–50% parasitemia (maximum parasitemia: 42.3% in the SE36/AHG group and 47.6% and 47.8% in the

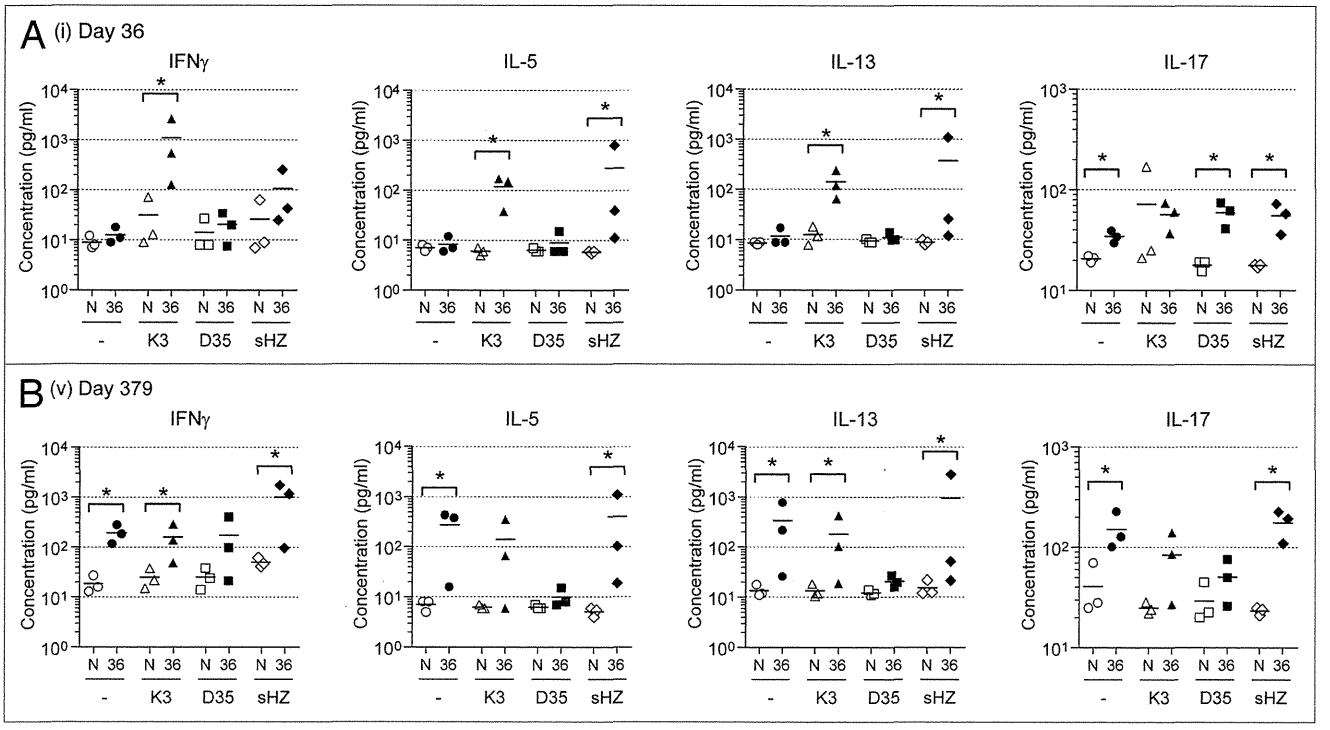

Figure 2. Cytokine response of PBMCs after stimulation with SE36 antigen in vitro. Levels in an individual cynomolgus monkey's cytokine (IFN-γ, IL-5, IL13 and IL-17) responses on days 36 (i) and 379 (v) are shown. "N" and "36" refer to non-stimulated (open symbols), and stimulated with SE36 (closed symbols), respectively. Closed circles, triangles, squares and diamonds indicate SE36/AHG, SE36/AHG with K3 ODN, SE36/AHG with D35 ODN and SE36/AHG with sHZ, respectively. Statistical analysis between pre- and post-immunization serum was performed using a Mann-Whitney U test. * indicates significant difference from the non-stimulated group, $p < 0.05$.

AHG with K3 ODN group) and were euthanized by Day 9 after challenge infection (**Fig. 4B**). These results indicate that the combined SE36/AHG with K3 ODN formulation afforded a level of protection that could substantially inhibit parasite growth. In the absence of robust statistical power, additional characterization of the induced anti-SE36 antibodies by the different treatments would be useful and could provide support for the observed correlation in the boosting of SE36-specific IgG titers after challenge infection and inhibition of parasite growth. Further in vitro assays would need to be incorporated in future studies.

To observe the importance of the anti-SE36 IgG antibody response, IgG titers against whole malaria parasite antigens were measured. For this purpose IgG titers against crude extract were measured after challenge infection. The IgG titers were immediately enhanced in all monkeys after challenge infection in contrast to responses of the anti-SE36 IgG titers (Tougan et al., unpublished data).

No serious adverse events during the studies were observed in both cynomolgus and squirrel monkeys. Common adverse reactions related to K3 ODN, such as erythema, swelling, induration or pruritus, were rarely detected at administration sites.

Discussion

Immunostimulatory CpG ODNs and sHZ that effect human immune cells in vitro often have limited immune-activating properties in mice. Relevant animal models such as non-human primates allow us the opportunity to demonstrate the safety and adjuvant activity of CpG ODNs and sHZ in vivo. In this study, we used cynomolgus and squirrel monkeys to evaluate the adjuvant efficacy of three TLR9 ligand adjuvants. In cynomolgus monkeys, all combined formulations of adjuvants with SE36/AHG enhanced SE36-specific IgG responses. In particular, the formulation with K3 ODN elicited over a 10-fold difference compared with SE36/AHG alone (**Fig. 1**). In **Figure 2**, it appears that formulations with K3 ODN enhanced the functions of helper T cells compared with those observed for SE36/AHG alone.

To date, many studies have demonstrated that various cytokines modulate an immune response during malaria infection. An increase in pro-inflammatory Th1 cytokines, such as IFN-γ and IL-12, during the acute phase of uncomplicated falciparum malaria has been inferred to play roles that contribute to an early and effective immune response, limiting progression toward a more severe course of malaria in humans.[26] Vaccine formulations containing CpG ODNs predominantly enhance Th1-associated cytokines, but Th2 responses involving IL-4 and IL-13 are often associated with AHG in mice.[16,27-30] However, the influence of CpG ODNs on cytokine responses in non-human primates has not been well characterized. In the current study, it was observed that the SE36/AHG formulation containing K3 ODN induced mixed Th1/Th2 responses in cynomolgus monkeys (**Fig. 2**).

The possibility of fluctuating T cell responses during this vaccination period is not clear as we did not perform cytokine assays on Days 112 (iii), 101 (ii), 365 (iv) or 583 (vi). Although this limitation would need to be addressed in future studies, this phenomenon has also been reported in several malaria vaccine candidates in mice, including AMA1-C1/AHG formulated with CPG 7909.[31] and SPf66-loaded PLGA microparticles.[32] A vaccination boost with recombinant SERA protein was also shown to markedly increase serum antibody titers in mice that were previously immunized with SERA plasmid DNA by gene gun vaccination.[33] In a murine malaria vaccine model, administration of *P. yoelii* $MSP1_{19}$/AHG formulated with CPG 7909, which is K-type CpG, induced a mixed Th1/Th2 response that resulted in enhanced vaccine efficacy, suggesting that the mixed Th1/Th2 response confers protective immunity against blood-stage infection.[34]

SE36-specific IgG titers were generally enhanced in all individual monkeys after injection of crude extract (**Fig. 3**). SE36 recombinant protein is derived from the N-terminal region of SERA5 based on the Honduras-1 strain corresponding to amino acids 17–382 where the serine repeats were removed (deletion of amino acids 193–225).[15] Although increased anti-SE36 IgG titers among all groups were not statistically different (**Fig. 3**), from squirrel monkey trials the formulation of K3 ODN appears effective for growth inhibition in various strains (**Fig. 4**).

In squirrel monkeys, the formulation with K3 ODN did not result in a higher antibody titer compared with the original SE36/AHG formulation, although protection that correlates with decreased parasite density in *P. falciparum* challenge was observed (**Fig. 4A and B**). The measurement of various cytokines in squirrel monkeys is not a sufficient measure of efficacy because they exhibit low levels of cross-reactivity with human cytokines.[35] Therefore, the interpretation of protective efficacy against malaria growth in terms of immunological responses is currently limited. Because the squirrel monkey is a valuable animal model for malaria vaccine development, the establishment of immunological analysis systems must provide more robust information for understanding the correlation between protective immunity and malaria infection.

No serious adverse events during the studies were observed in both cynomolgus and squirrel monkeys. Common adverse reactions related to K3 ODN were rarely detected at administration sites. Although empirical, the K3 ODN adjuvant formulation exhibited an adequate safety profile, justifying further studies to evaluate humoral and cellular responses in humans. A number of clinical trials looking at CpG ODN formulations have been initiated in the field of malaria vaccine development. Formulations of K-type ODN, CpG 7909, with MSP1 and AMA1 effectively boost antigen-specific IgG levels and demonstrate an adequate safety profile.[36-39] In clinical trials for hepatitis B (Engerix-B) and flu (Fluarix) vaccines, enhanced safety and immunogenicity of CpG 7909 formulations have been reported.[40,41]

In conclusion, our study demonstrates that the formulation of K3 ODN with SE36/AHG can result in the improvement of immune response.

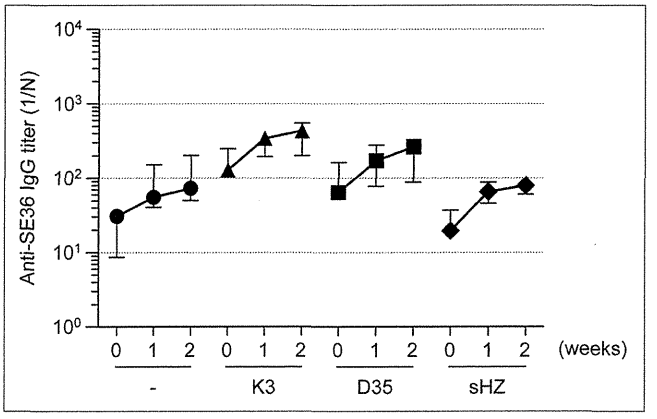

Figure 3. Trends in SE36-specific IgG antibody titers from cynomolgus monkeys after the mimic challenge infection. Crude extract was administered on day 583, and sera from each monkey were collected on day 583 (week 0), weeks 1 and 2. Median titers are represented by closed circles (SE36/AHG), triangles (SE36/AHG with K3 ODN), squares (SE36/AHG with D35 ODN) and diamonds (SE36/AHG with sHZ). Ranges are shown by bars. Statistical analysis at each timepoints (week 1 and 2) was performed using non-parametric ANOVA (Kruskal-Wallis) with Dunn's post-hoc test.

Materials and Methods

Animals, immunization and infection. A total of 12 cynomolgus monkeys (*Macaca fascicularis*) were obtained from Tsukuba Primate Research Center (TPRC), National Institute of Biomedical Innovation (NIBIO) and randomly assigned to four groups (n = 3/group). Animals were immunized subcutaneously on days 0, 22, 101 and 365 of our study with a mixture of 10 μg of SE36 and 125 μg of AHG with and without 500 μg of K3 ODN or D35 ODN in a total volume of 1 ml, or with 1.5 mM sHZ in a total volume of 1 ml. The SE36 antigen and AHG adjuvant were GMP-quality[15] and K3 and D35 ODNs and sHZ adjuvants were prepared specifically for this study. K3 and D35 were prepared as GMP quality by Gene Design Inc. and sHZ was prepared under sterile conditions with non-detectable endotoxin levels determined by LAL assay.

A total of seven squirrel monkeys (*Saimiri sciureus*) were purchased from PETSUN Co., Ltd. and randomly assigned to three groups: SE36/AHG (n = 2); SE36/AHG with K3 ODN (n = 3); and AHG with K3 ODN group (n = 2). These animals were subcutaneously immunized twice at a 3-week interval with a combination of 10 μg of SE36, 125 μg of AHG with or without 500 μg of K3 ODN in a total volume of 0.5 ml. The monkeys were splenectomized before inoculation. Nine weeks after the first immunization, parasite challenge experiments were done using the live IPC/Ray strain[42] (5×10^8 infected red blood cells) introduced intravenously in the saphenous vein. Parasitemia was monitored daily by counting 5,000 RBCs in Giemsa-stained thin blood smears. Drug treatment was commenced at Day 14, and in cases where parasitemia surpassed the set threshold parasitemia (40%) during the experimental period, the animal was euthanized to avoid sequelae, such as severe malaria, in accordance with the guidelines for animal welfare and care (Guidelines

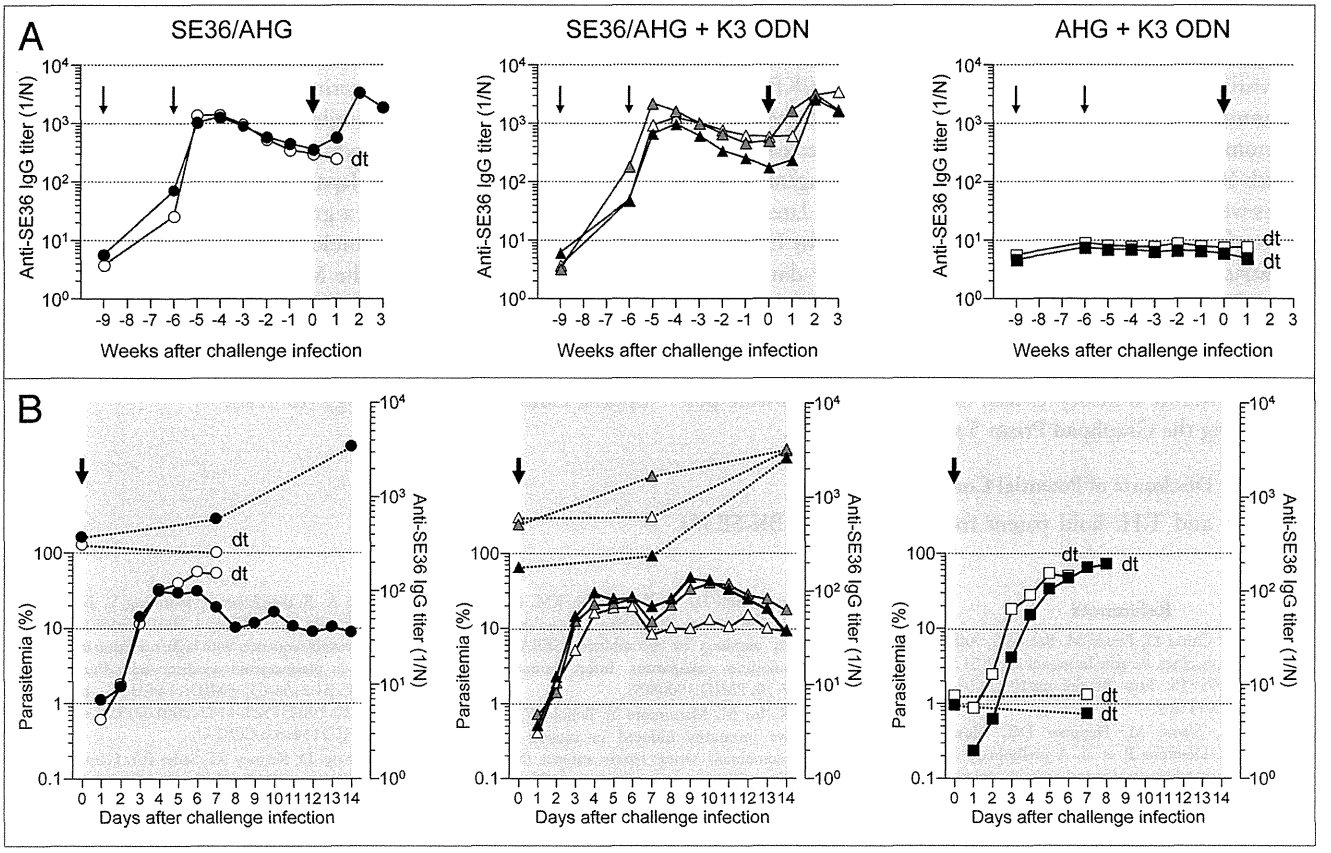

Figure 4. Vaccine trial in squirrel monkeys. (**A**) Time-course analysis of SE36-specific IgG antibody titer. Animals were administered 10 μg of SE36, 100 μg of AHG and/or 500 μg of K3 ODN at 6 and 9 weeks (thin arrows) before the challenge infection (bold arrow). (**B**) Time-course analysis of parasitemia. Parasite densities were measured up to 14 d after infection. Left Y-axis shows parasitemia and right Y-axis shows SE36-specific IgG antibody titers. Bold arrow shows the challenge infection. Dotted lines show the SE36-specific IgG antibody titers as in (**A**). In (**A**) and (**B**), light gray area shows duration of parasitemia measurement. The term "dt" denotes a monkey that was euthanized, or died owing to acute conditions. Groups are represented by closed circles (SE36/AHG), triangles (SE36/AHG with K3 ODN) and squares (AHG with K3 ODN). Black, gray and white symbols indicate the individual animals in each group.

for the Animal Care and Management of TPRC, NIBIO). All experimental protocols were approved by the Animal Welfare and Animal Care Committee at TPRC, NIBIO where animals were housed and handled. The vaccination schedule was identical to GLP studies and clinical trials for SE36 malaria vaccine.[15]

All experimental protocols were approved by the Animal Welfare and Animal Care Committee at TPRC, NIBIO. All animals were housed and handled in accordance with the Guidelines for Laboratory Animals at TPRC, NIBIO.

Adjuvants. The following human-type CpG ODNs were used: K3 ODN (ATCGACTCTCGAGCGTTCTC) and D35 ODN (GGtgcatcgatgcaggggGG). Bases shown in uppercase are phosphorothioate, and those in lowercase are phosphodiester, with CpG dinucleotides underlined.[20] These ODNs were purchased from GeneDesign Inc. The sHZ was purified from hemin chloride using the acid-catalyzed method to produce smaller and homogenous crystals as previously described.[24,25,43,44]

Antibody detection by indirect enzyme-linked immunosorbent assay (ELISA). An optimized concentration (1 μg/ml) of the recombinant SE36 antigen in carbonate/bi-carbonate buffer (pH 9.6) was used to coat wells of a 96-well MaxiSorp NUNC-Immuno plate (Nunc, Catalog number: 442404) overnight at 4°C. Plates were washed and blocked with 5% skim milk in phosphate-buffered saline (PBS) containing 0.05% Tween 20 for 2 h at room temperature. Cynomolgus monkey IgG was detected using horseradish peroxidase (HRP)-conjugated anti-monkey IgG (whole molecule) antibody produced in rabbit (Sigma-Aldrich, Catalog number: A2054). Squirrel monkey IgG was detected using anti-squirrel monkey IgG polyclonal antibody produced in rat (a kind gift from Dr. T. Tachibana) and HRP-conjugated anti-rat IgG (whole molecule) antibody produced in rabbit (Sigma-Aldrich, Catalog number: A5795). Color was developed using a TMB microwell peroxidase substrate (KPL, Catalog number: 50–76–00). The reaction was stopped with 1 M H_2SO_4. Absorbance was measured at 450/540 nm within 30 min using a SpectraMax 340PC^{384} Microplate Reader (Molecular Devices).

Detection of Cytokines. PBMCs of cynomolgus monkeys were purified by Ficoll-Hypaque (GE Healthcare, Catalog number: 17–1440–02) gradient centrifugation from blood samples obtained at days 36 and 378. Isolated cells were stimulated with GMP-quality SE36 antigen or PBS at 37°C for 24 h. Cytokine

concentrations in the culture supernatant were determined using a Milliplex Non-Human Primate Cytokine Panel - Premixed 23 Plex (Millipore, Catalog number: MPXPRCYTO40KPX23).

Immunostimulation using crude extracts of *P. falciparum*. All cynomolgus monkeys were subcutaneously injected on Day 583 with 100 μl of crude 3D7 parasite lysate (21.5 mg/ml). Blood samples were drawn at 0 (Day 583), 1 and 2 weeks later and the amount of SE36-specific IgG antibodies measured by ELISA.

Statistical analysis. Comparisons of IgG titer data among groups of monkeys were performed by non-parametric ANOVA (Kruskal-Wallis) with Dunn's post-hoc test. Comparisons of cytokine levels between two groups were performed by non-parametric Mann-Whitney U test. Statistical analyses were performed using the Graphpad Prism 5 software package.

Disclosure of Potential Conflicts of Interest

T.T., K.J.I. and T.H. hold patent for production of BK-SE36/CpG.

Acknowledgments

We thank Dr. Taro Tachibana at Department of Bioengineering, Graduate School of Engineering, Osaka City University for anti-squirrel monkey IgG antibody. This work was supported by the New Energy and Industrial Technology Development Organization (NEDO) of Japan (97S08–011 to T.H.). This work was also supported by a grant from the Cooperative Link of Unique Science and Technology for Economy Revitalization (CLUSTER) promoted by the Ministry of Education, Culture, Sports and Technology (to T.H. and K.J.I.). And this work was partially supported by Grant-in-Aid for Young Scientists (B) (22700455) from the Japanese Ministry of Education, Science, Sports, Culture and Technology (to T.T.).

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Volume 33 Supplement 2 2013