SOCS3 expression, which inhibits the Th1 and Th17 differentiation through the inhibition of the STAT3 and STAT4 phosphorylation.

Pitavastatin Regulates Th1 and Th17 Differentiation in an Isoprenylation-Dependent Manner

Next, we assessed the effect of pitavastatin on the Th differentiation. Pitavastatin-treated T cells failed to differentiate into IFN-γ-producing Th1 or IL-17-producing Th17 cells under the appropriate skewing conditions in vitro (Fig. 3a). Pitavastatin blunted the production of IFN-γ and IL-17 in the culture supernatant, which was reversed by mevalonate supplement (Fig. 3b). Thus pitavastatin interferes with the Th1 and Th17 differentiation.

As a result of the HMG-CoA reductase inhibition, statins regulate the biosynthesis of mevalonate pathway-derived isoprenoids, leading to reduced isoprenylation of protein targets (Fig. 4a). To confirm the involvement of isoprenylated proteins in the T-cell differentiation, we investigated the effects of a farnesyltransferase inhibitor (FTI-277) and a geranylgeranyltransferase inhibitor (GGTI-298) on $CD4^+$ T cells. Under Th1 skewing conditions, both FTI-277 and GGTI-298 inhibited the Th1 differentiation (Fig. 4b and c). In contrast, FTI-277 inhibited the Th17 differentiation, whereas GGTI-298 did not (Fig. 4b and c). We also examined whether farnesyl-PP and geranylgeranyl-PP could reverse the inhibitory effects of pitavastatin on the Th1/Th17 differentiation. As shown in Fig. 3b, farnesyl-PP reversed the inhibitory effects of pitavastatin on both the Th1 and Th17 differentiation, however, geranylgeranyl-PP only did so on the Th1 differentiation. Collectively, these results suggest that pitavastatin prevents Th1 and Th17 differentiation by inhibiting the biosynthesis of isoprenoids.

Pitavastatin Influenced the Expression of Transcription Factors in T Cells

The transcription factors, T-bet and RORγT, have critical roles in the development of Th1 and Th17 cells, respectively [28–30]. Next we sought to determine if these transcription factors were affected by pitavastatin. Pitavastatin-treated T cells

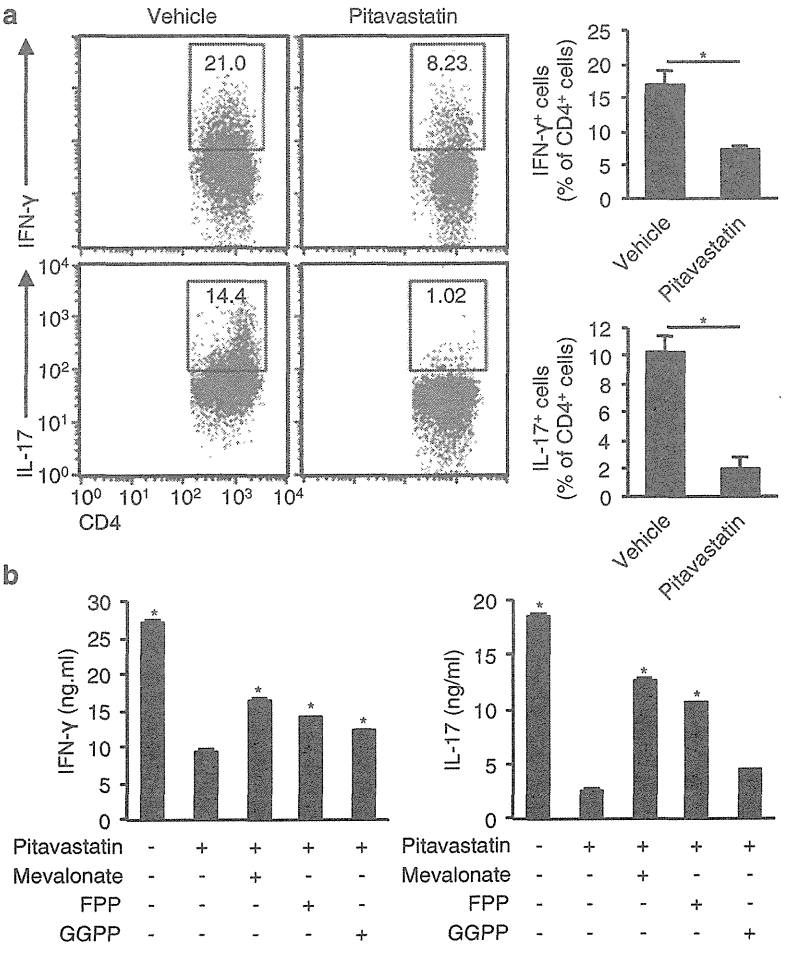

Fig. 3 Pitavastatin inhibits the Th1 and Th17 differentiation. **a** The IFN-γ and IL-17 production in $CD4^+$ T cells treated with pitavastatin overnight, followed by 2 d of stimulation with anti-CD3 and anti-CD28 in Th1- or Th17-skewing conditions. The numbers in the outlined areas indicate the percent of cytokine-producing $CD4^+$ T cells. **b** The IFN-γ and IL-17 concentration in the culture supernatant of $CD4^+$ T cells treated with pitavastatin with or without mevalonate, farnesyl-PP (FPP) or geranylgeranyl-PP (GGPP) overnight, followed by 2 d of stimulation as in **a**. Data are expressed as the mean ± SEM from triplicate culture wells. The data are from 1 of 2 experiments performed, with similar results. $*P<0.05$

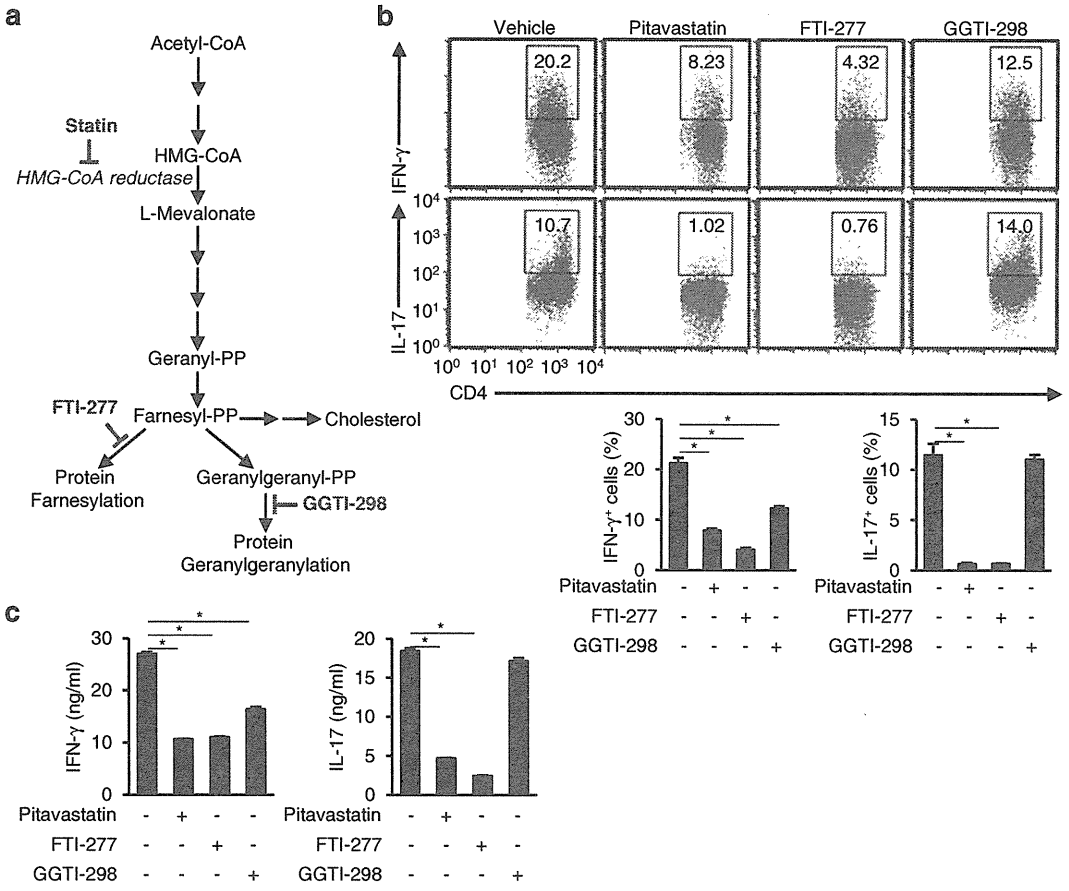

Fig. 4 Inhibitors of isoprenylation prevent the Th1 and Th17 differentiation. **a** The mevalonate pathway. Drug inhibitors are shown in red. Not all pathway intermediates are shown. **b** The IFN-γ and IL-17 production in $CD4^+$ T cells treated with pitavastatin, farnesyltransferase inhibitors (FTI-277) or geranylgeranyltransferase inhibitors (GGTI-298) overnight, followed by 2 d of stimulation as in Fig. 3a. The numbers in the outlined areas indicate the percent of cytokine-producing $CD4^+$ cells. **c** The IFN-γ and IL-17 accumulation in the culture supernatant of $CD4^+$ T cells treated as in **b**. Data are expressed as the mean ± SEM from triplicate culture wells. The results of one of two representative experiments are shown. $^*P<0.05$

failed to fully upregulate the T-bet mRNA (*Tbx21*) and protein under Th1 conditions and the RORγT mRNA (*Rorc*) and protein under Th17 conditions (Fig. 5a and b). Mevalonate reversed the effects of pitavastatin on the expression of these transcription factors (Fig. 5a and b). To confirm the involvement of isoprenylated proteins in the expression of transcription factors, we investigated the effects of FTI-277 and GGTI-298 on $CD4^+$ T cells. As shown in the Supplementary Figure, FTI-277 suppressed both the T-bet and RORγT, whereas GGTI-298 only suppressed the T-bet expression. These results suggested that pitavastatin affected the Th1 and Th17 differentiation through inhibiting the T-bet and RORγT transcription.

Adoptive Transfer of Pitavastatin-Treated $CD4^+$ T Cells Prevents Induction of EAM in Recipient Mice

If the myocarditis resistance of pitavastatin-treated mice indeed results from the inhibition of the Th differentiation, an adoptive transfer of pitavastatin-treated autoreactive $CD4^+$ T cells would fail to induce myocarditis in the recipient mice. To test this hypothesis, we isolated $CD4^+$ T cells from EAM mice either given or not given pitavastatin treatment and transferred them into SCID mice. All of the recipient mice that received $CD4^+$ T cells from vehicle-treated mice developed severe myocarditis (Fig. 6a and b). In contrast, mice that received donor $CD4^+$ T cells from mice treated with pitavastatin were protected from severe myocarditis.

In order to test whether in vitro pitavastatin treatment affects the effector T-cell function, we isolated $CD4^+$ T cells from EAM mice and treated them with pitavastatin or FTI-277 in vitro. Pitavastatin and FTI-277 inhibited the MyHC-α-specific IFN-γ and IL-17 production (Fig. 6c). We also found that in vitro pitavastatin-treated $CD4^+$ T cells failed to induce myocarditis in the recipients (Fig. 6d and e). These data suggest that pitavastatin was able to impair not only the priming but also the effector phases of T-cell responses, and

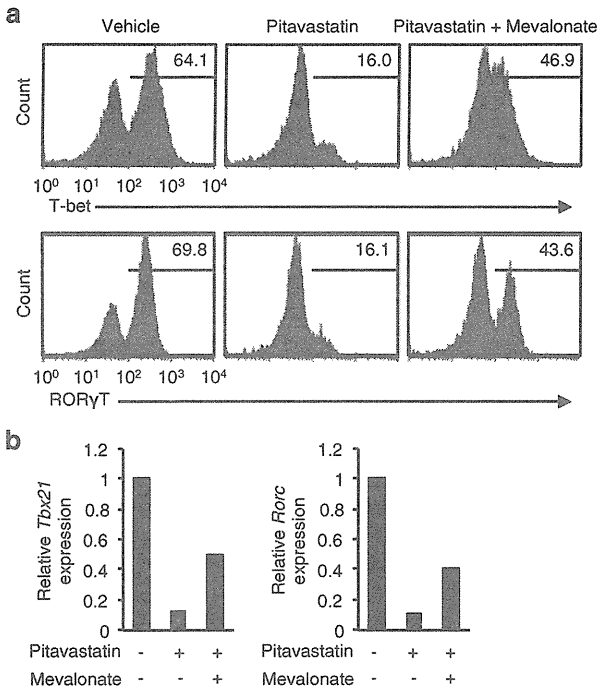

Fig. 5 Control of the induction of transcription factors by pitavastatin. **a** T-bet and RORγT expression in $CD4^+$ T cells treated with pitavastatin with or without mevalonate overnight, followed by 2 d of stimulation with anti-CD3 and anti-CD28 in Th1- or Th17-skewing conditions. The numbers indicate the percentage of transcription factor-expressing $CD4^+$ cells. **b** The *Tbx21* and *Rorc* mRNA expression in $CD4^+$ T cells treated as in a. The data were normalized for the basal gene expression in vehicle-treated cells. The results of one of two representative experiments are shown

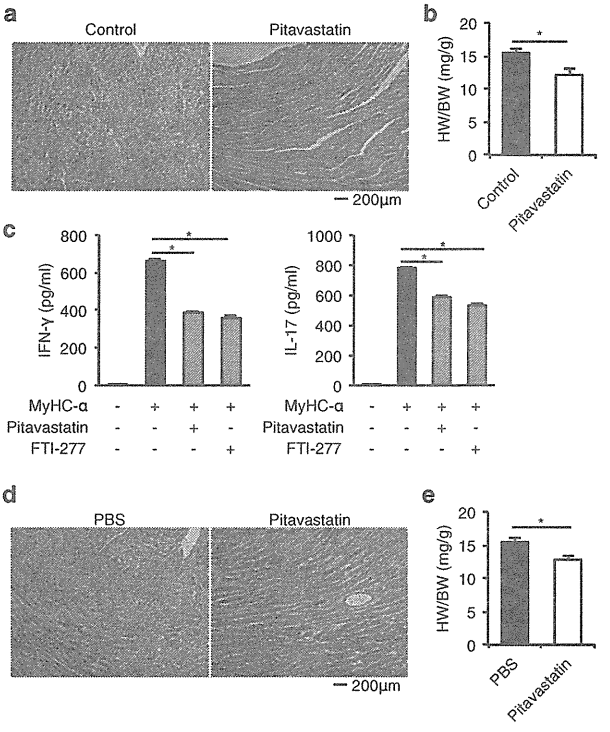

Fig. 6 Adoptive transfer of in vivo or in vitro pitavastatin-treated $CD4^+$ T cells prevents the induction of EAM in recipient mice. **a** and **b** 5×10^6 $CD4^+$ T cells were isolated from vehicle- or pitavastatin-treated EAM mice and restimulated with 5 μg/ml of MyHC-α in the presence of APCs for 2 d, and then transferred into SCID mice (n=5 per group). H&E-stained heart sections 10 d after transfer (**a**) and the heart-to-body weight ratios of the recipient mice (**b**). **c** $CD4^+$ T cells were isolated from EAM mice and treated with the indicated agents in the presence of APCs for 2 d in vitro. **d** and **e** 5×10^6 $CD4^+$ T cells were isolated from EAM mice and restimulated with MyHC-α and APCs in the presence or absence of pitavastatin for 2 d, and then transferred into SCID mice (n=5 per group). H&E-stained heart sections 10 d after transfer (**d**) and the heart-to-body weight ratios of the recipient mice (**e**). Data are expressed as the mean ± SEM from triplicate culture wells. The results of one of two representative experiments are shown. *P<0.05

these adoptive transfer studies provided evidence that pitavastatin treatment impaired the $CD4^+$ T-cell function that suppressed clinical autoimmune myocarditis.

Discussion

As a result of the HMG-CoA reductase inhibition, statins inhibit the synthesis of mevalonate pathway-derived isoprenoids (Fig. 4a). In the present study, we provide evidence that a blockade of this enzyme by pitavastatin inhibits Th1 and Th17 responses through the inhibition of the protein isoprenylation. Pitavastatin-treated T cells failed to differentiate into Th1 and Th17 cells, and this failure was rescued by adding mevalonate. In a mouse model of EAM, pitavastatin treatment ameliorated the pathophysiological severity of myocarditis associated with reduced Th1 and Th17 responses. Our results are important for understanding the anti-inflammatory effects of statins.

Myocarditis and subsequent dilated cardiomyopathy (DCM) are major causes of heart failure in young patients. The activation and differentiation of T cells play a critical role in the pathogenesis of both situations [31]. Although there are several reports on the effect of statins on T-cell cytokine secretion in EAE, there are few studies on EAM. Liu et al. reported that atorvastatin ameliorated EAM in rats, attributing it to a shift from Th1 to Th2 cytokine secretion [32]. Our study examined the effect of statins on the Th17 response in EAM, which has not been explored before. In the present study, we found that both in vivo and in vitro treatment with pitavastatin inhibited the Th1 and Th17 cytokine production from cardiac-specific $CD4^+$ T cells, which was associated with the disease severity. Thus, pitavastatin can directly modulate the T-cell function and reduce the pathogenicity of T cells confirmed by adoptive transfer experiments.

EAM represents a $CD4^+$ T cell-mediated disease. Th1 cells were once considered the major pathogenic subset mediating

organ-specific autoimmune diseases [33, 34]. However, IFN-γ has recently been shown to be a down-regulatory cytokine, as evidenced by exacerbated myocarditis in IFN-γ receptor knockout (KO), IFN-γ KO, and T-bet KO mice [35–37]. On the other hand, Th17 cells have been implicated in the pathogenesis of various types of autoimmune diseases (reviewed in [38]); however, the genetic ablation of IL-17 had no significant impact on the incidence or severity of myocarditis [39]. These gene-ablated mice provided us with much important information, and we could not exclude the possibility that the inhibitory effect of pitavastatin on the IFN-γ production may weaken the immunosuppressive effect of pitavastatin. However, studies from gene ablation mice do not necessarily match the results of the real world. We have previously reported that the suppressor of cytokine signaling 1 (SOCS1) DNA administration ameliorated EAM, and SOCS1 DNA therapy suppressed both Th1 and Th17 cytokines from $CD4^+$ T cells [10]. Ohshima et al. also reported that systemic transplantation of allogenic fetal membrane-derived mesenchymal stem cells suppresses both Th1 and Th17 T-cell responses in EAM [40]. The question of the relative roles of Th1 cells versus Th17 cells on EAM is unresolved [39, 41], and autoimmune diseases have so far been considered to be associated with both Th1 and Th17 cells [39, 42]. Statins can inhibit both Th1 and Th17 responses and may be a useful therapy for autoimmune diseases in the clinical setting.

As shown in Fig. 2a, a larger amount of pitavastatin was needed to inhibit STAT3 phosphorylation than was needed to inhibit STAT4 phosphorylation. Kagami et al. showed similar results that simvastatin inhibited the differentiation of Th17 cells at the concentrations that did not inhibit the differentiation of Th1 cells [43]. These findings suggest that statins can more easily affect the Th17 differentiation than affect the Th1 differentiation.

Several studies have shown that statins can modulate the inflammatory milieu by altering chemokines and chemokine receptors, eventually inhibiting the leukocyte migration [44, 45]. We evaluated the chemokine concentrations in the heart and found decreased levels of chemokines, including CCL2, CCL3, CCL5, CCL17, CCL20 and CXCL10 (Fig. 1e). We could not evaluate whether these decreases were direct effects of pitavastatin or not in our system, and this may have contributed to the beneficial effects of pitavastatin in EAM.

There are several reports of the effects of statins on inducing Tregs in humans. Atorvastatin treatment of human PBMCs in vitro led to an induction of the transcription factor Foxp3, accompanied by an increase in the number of Tregs [46]. Simvastatin and pravastatin treatment in hyperlipidemic patients increased the number of Tregs in PBMCs [46]. Tang et al. showed that atorvastatin promoted the generation of Tregs from primary T cells of rheumatoid arthritis patients [47]. On the other hand, there are some conflicting data on the Treg induction by statins in mice. Consistent with our data (Fig. 2d), Mausner-Fainberg et al. did not find a statin-induced conversion of murine $CD4^+CD25^-Foxp3^-$ to Treg cells [46]. Mira et al. also demonstrated that no changes were noted in the Treg cell numbers in the spleen or draining lymph nodes of lovastatin-treated mice [48]. However, it has been reported that simvastatin increases the Treg differentiation in vitro [43, 49]. Thus, the question of the effect of statins on Tregs in mice is unresolved. It can be assumed that in murine models, the anti-inflammatory effects of pitavastatin are probably evident by inhibiting the Th1 and Th17 response rather than by the expansion of Tregs. Further studies are needed to elucidate the effect on the Treg induction in mice.

As a result of the HMG-CoA reductase inhibition, statins also inhibit the synthesis of downstream isoprenoids farnesyl-PP and geranylgeranyl-PP (Fig. 4a). The process of the isoprenylation of Ras/Rho family proteins is required for G protein activity. At least some of the pleiotropic benefits of statins that are independent of the cholesterol lowering effect are thought to involve interference with the normal synthesis of isoprenoids, thereby impairing the Ras/Rho G protein function (reviewed in ref. [50]). Dunn et al. showed that atorvastatin suppressed Th1 differentiation and caused a Th2 bias by inhibiting Ras farnesylation and RhoA geranylgeranylation [51]. Ras-ERK and RhoA-p38 signaling pathways are important in determination of Th1/Th2 fate [52–54]. In accordance with this evidence, our data have demonstrated that both inhibitors of farnesylation and geranylgeranylation prevented the Th1 differentiation (Fig. 4b and c). On the other hand, the Th17 differentiation was inhibited only by a farnesylation inhibitor (Fig. 4b and c). Recently, Rheb which belongs to the Ras family of G proteins [55] and downstream mammalian target of rapamycin (mTOR) signaling in T cells was shown to be necessary for Th1 and Th17 responses and the development of autoimmune disease [56]. Rheb-deficient T cells failed to differentiate into Th1 and Th17 cells, and mice with T cell-specific deletion of Rheb were resistant to the development of EAE [56]. Farnesylation of Rheb is necessary for its intracellular trafficking and subcellular localization to the plasma membrane and subsequent activation of the mTOR pathway [57]. Some in vitro studies have demonstrated an inhibitory effect of statin treatment on the farnesylation and membrane-association of Rheb [58, 59]. From our ongoing study, we have found that pitavastatin inhibited Rheb-mTOR signaling in a farnesylation-dependent manner (unpublished data). Collectively, these results suggest that pitavastatin prevents Th1 and Th17 differentiation by inhibiting the biosynthesis of farnesyl-PP, leading to reduced farnesylated Rheb and reduced downstream mTOR activity. Our data have demonstrated that both FTI-277 and GGTI-298 inhibited the Th1 differentiation, but Th17 differentiation was inhibited only by FTI-277 (Fig. 4). These results may have indicated that Th17 differentiation

is mainly regulated by farnesylated Rheb-mTOR signaling in our system, though Th1 differentiation is regulated not simply by farnesylated Rheb but also by other geranylgeranylated proteins. Further studies are needed to evaluate the mechanism.

In the present study, we have shown that both FTI-277 and GGTI-298 inhibited the Th1 differentiation, but the Th17 differentiation was inhibited mainly by FTI-277 (Fig. 4b and c). In contrast to our data, Kagami et al. reported that the inhibition of protein geranylgeranylation but not farnesylation is involved in the decreased differentiation of Th17 cells with simvastatin treatment [43]. There were some differences between their experiments and ours. They used a low-dose (5 μM) of FTI-277 and GGTI-298 in their experiments, whereas we used a relatively high amount of those reagents (20 μM). Therefore, we also checked the effects of low-dose FTI-277 and GGTI-298 on the Th differentiation and did not find any apparent effects on the Th differentiation (data not shown). Next, they used $CD4^+CD25^-$ T cells for the Th skewing experiments, whereas we used $CD4^+CD62L^+$ T cells. CD25 (IL-2 receptor α chain) has been used as a marker for Tregs as well as activated T cells [60]. On the other hand, CD62L (L-selectin) is an important T-cell homing receptor as well as a marker for T-cell development. Naive T cells are $CD62L^+$, and CD62L acts as a "homing receptor" for lymphocytes to enter secondary lymphoid tissues via high endothelial venules [61]. This difference may have influenced the results.

In conclusion, we have shown that pitavastatin inhibits $CD4^+$ T-cell proliferation and Th1/Th17 responses and ameliorates myocarditis in mice. Because oral statin administration is well tolerated, this treatment is a promising approach for the treatment of Th1- and Th17-mediated autoimmune diseases.

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Conflict of Interest The authors declare that they have no conflict of interest.

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ORIGINAL ARTICLE

Dynamics of cellular immune responses in the acute phase of dengue virus infection

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Abstract In this study, we examined the dynamics of cellular immune responses in the acute phase of dengue virus (DENV) infection in a marmoset model. Here, we found that DENV infection in marmosets greatly induced responses of CD4/CD8 central memory T and NKT cells. Interestingly, the strength of the immune response was greater in animals infected with a dengue fever strain than in those infected with a dengue hemorrhagic fever strain of DENV. In contrast, when animals were re-challenged with the same DENV strain used for primary infection, the neutralizing antibody induced appeared to play a critical role in sterilizing inhibition against viral replication, resulting in strong but delayed responses of CD4/CD8 central memory T and NKT cells. The results in this study may help to better understand the dynamics of cellular and humoral immune responses in the control of DENV infection.

T. Yoshida and T. Omatsu contributed equally to this study.

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Introduction

Dengue virus (DENV) causes the most prevalent arthropod-borne viral infections in the world [29]. Infection with one of the four serotypes of DENV can lead to dengue fever (DF) and sometimes to fatal dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) [12]. The serious diseases are more likely to develop after secondary infection with a serotype of DENV that is different from that of the primary infection. Infection with DENV induces a high-titered neutralizing antibody response that can provide long-term immunity to the homologous DENV serotype, while the effect of the antibody on the heterologous serotypes is transient [22]. On the other hand, enhanced pathogenicity after secondary DENV infection appears to be explained by antibody-dependent enhancement (ADE). Mouse and monkey experiments have shown that sub-neutralizing levels of DENV-specific antibodies actually enhance infection [1, 6, 11]. Thus, the development of an effective tetravalent dengue vaccine is considered to be an important public-health priority. Recently, several DENV vaccine candidates have undergone clinical trials, and most of them target the induction of neutralizing antibodies [20].

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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

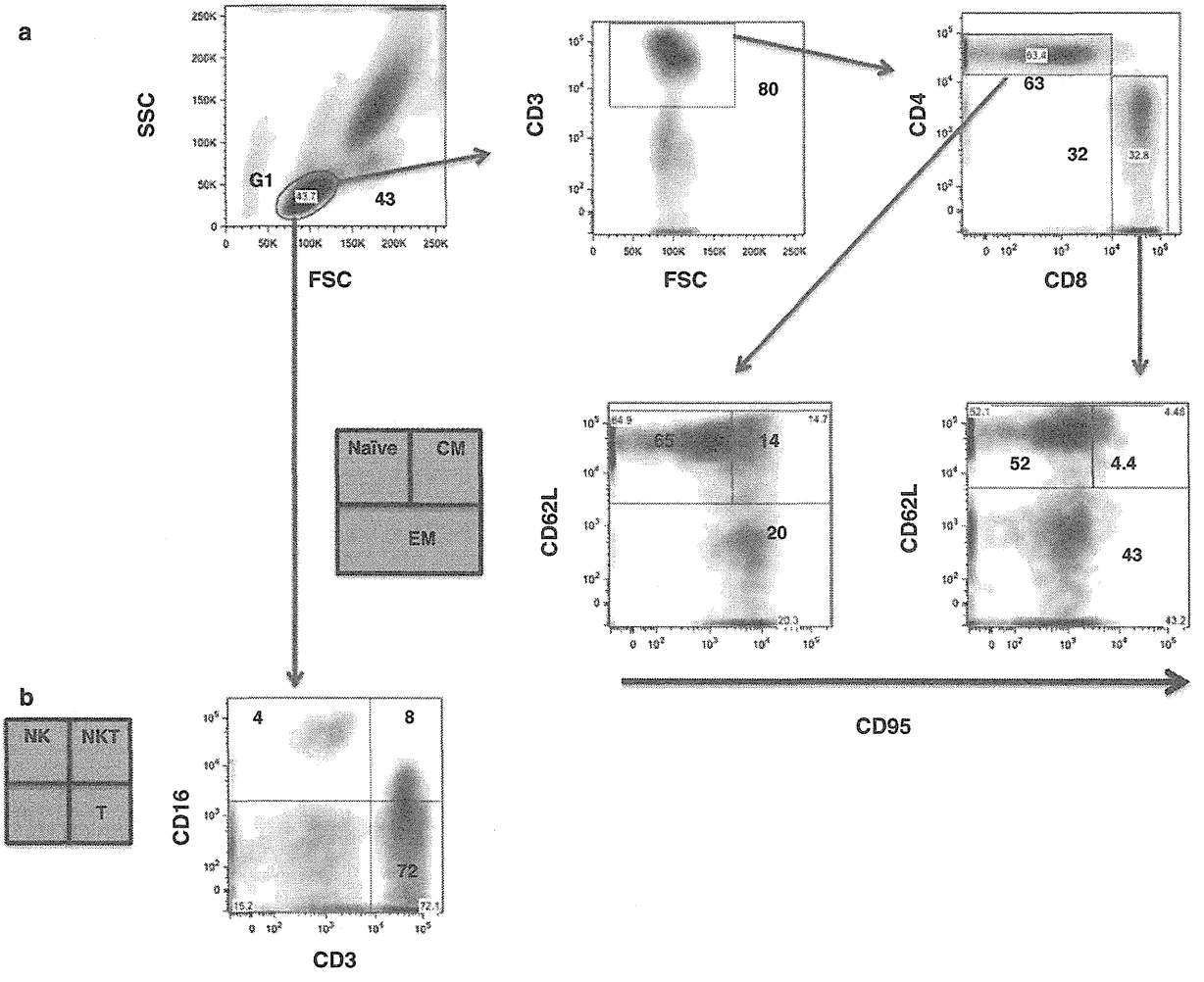

Fig. 1 Flow cytometric analysis of naïve, central/effector memory T cells and NK/NKT cells in marmosets. (**a**) Gating strategy to indentify CD4 and CD8 T, NK and NKT cells. The G1 population was selected and analyzed for CD4 and CD8 T, NK and NKT cells. (**a**) Profiling of naïve, central memory, and 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

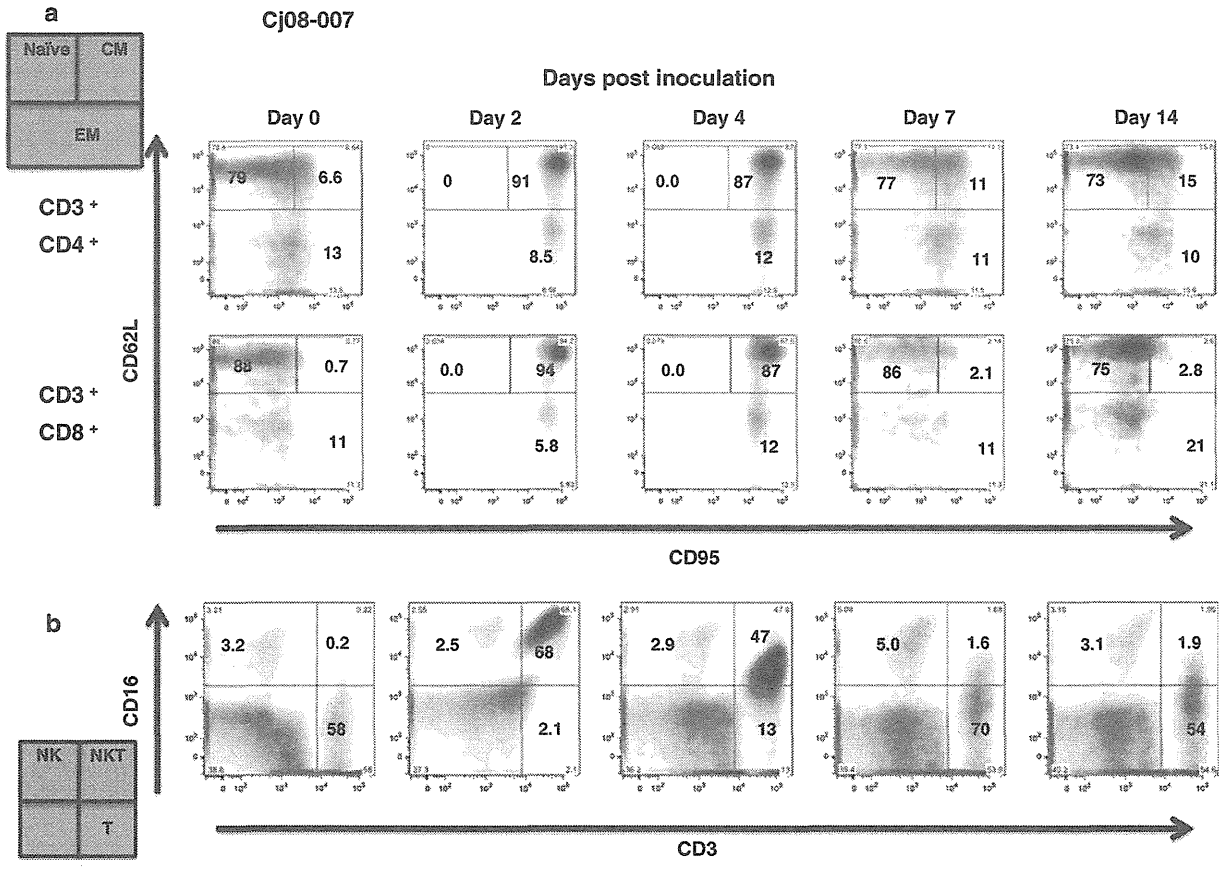

Fig. 2 Profiling of CD4 and CD8 T, NK and NKT cells in marmosets with primary infection with the DENV-2 Mal/77/08 strain. For primary DENV infection, two marmosets were inoculated subcutaneously in the back with 1.9×10^5 PFU of the DENV-2 Mal/77/08 strain. (**a**) Profiling of naïve, central memory,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

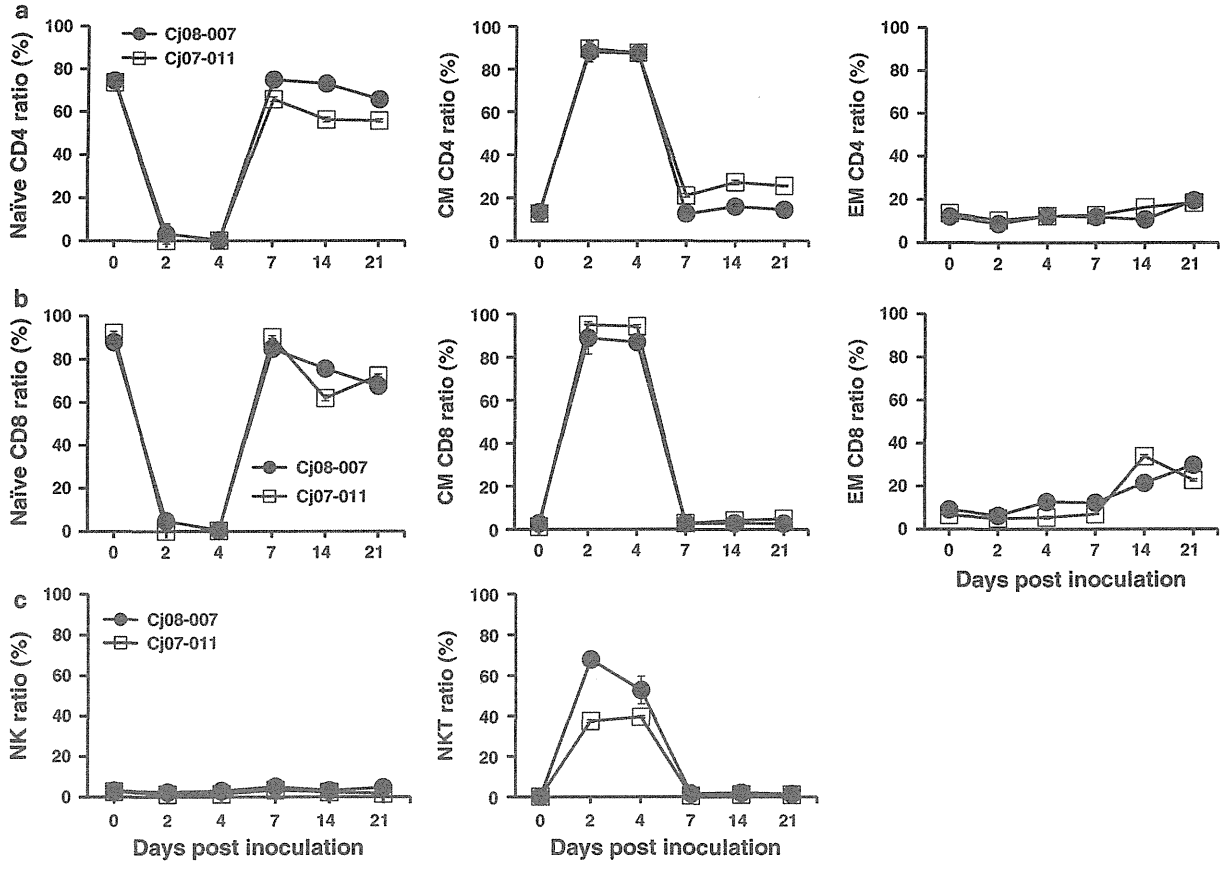

Fig. 3 Frequency of CD4 and CD8 T, NK and NKT cells in marmosets with primary infection with the DENV-2 Mal/77/08 strain. For primary DENV infection, two marmosets were inoculated subcutaneously in the back with 1.9×10^5 PFU of the DENV-2 Mal/77/08 strain. (a) Ratios of naïve, central memory, and effector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

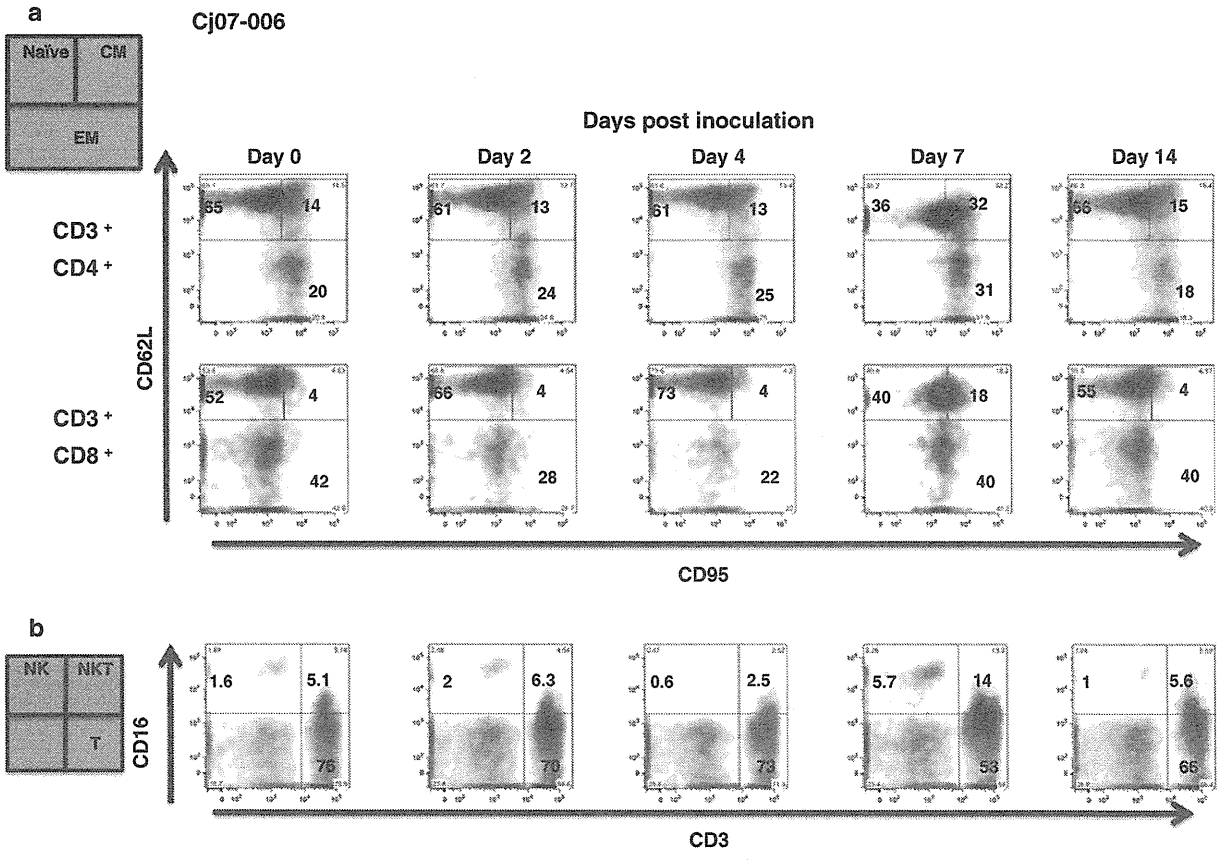

Fig. 4 Profiling of CD4 and CD8 T, NK and NKT cells in marmosets with primary infection with the DENV-2 DHF0663 strain. For primary DENV infection, two marmosets were inoculated subcutaneously in the back with 1.8×10^4 PFU of the DENV-2 DHF0663 strain. (**a**) Profiling of naïve, central memory, and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

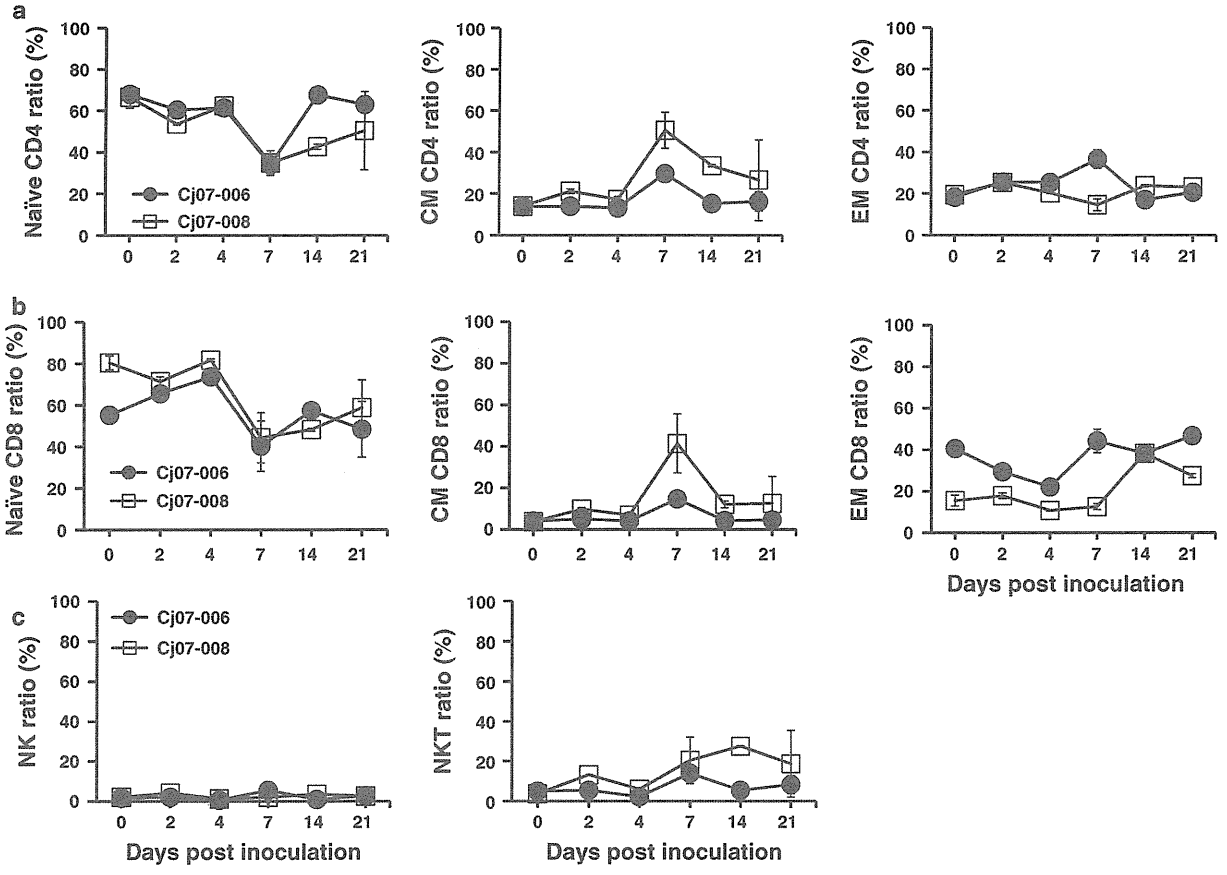

Fig. 5 Frequency of CD4 and CD8 T, NK and NKT cells in marmosets with primary infection with the DENV-2 DHF0663 strain. For primary DENV infection, two marmosets were inoculated subcutaneously in the back with 1.8×10^4 PFU of the DENV-2 DHF0663 strain. (**a**) Ratios of naïve, central memory, and effector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

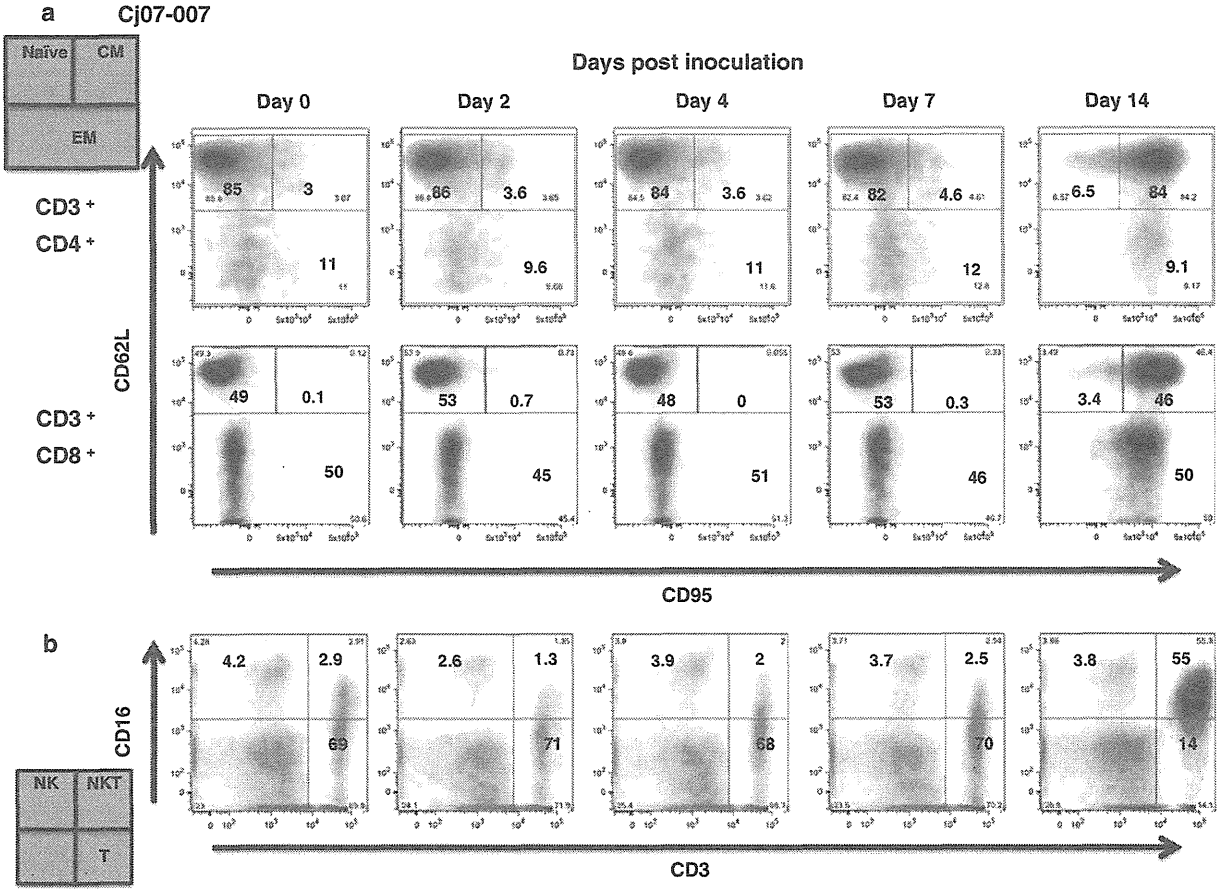

Fig. 6 Profiling of CD4 and CD8 T, NK and NKT cells in marmosets after re-challenge with the DENV-2 DHF0663 strain. Two marmosets that were initially inoculated with 1.8×10^5 PFU of the DHF0663 strain were re-inoculated 33 weeks after the primary challenge with 1.8×10^5 PFU of the same strain. (**a**)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

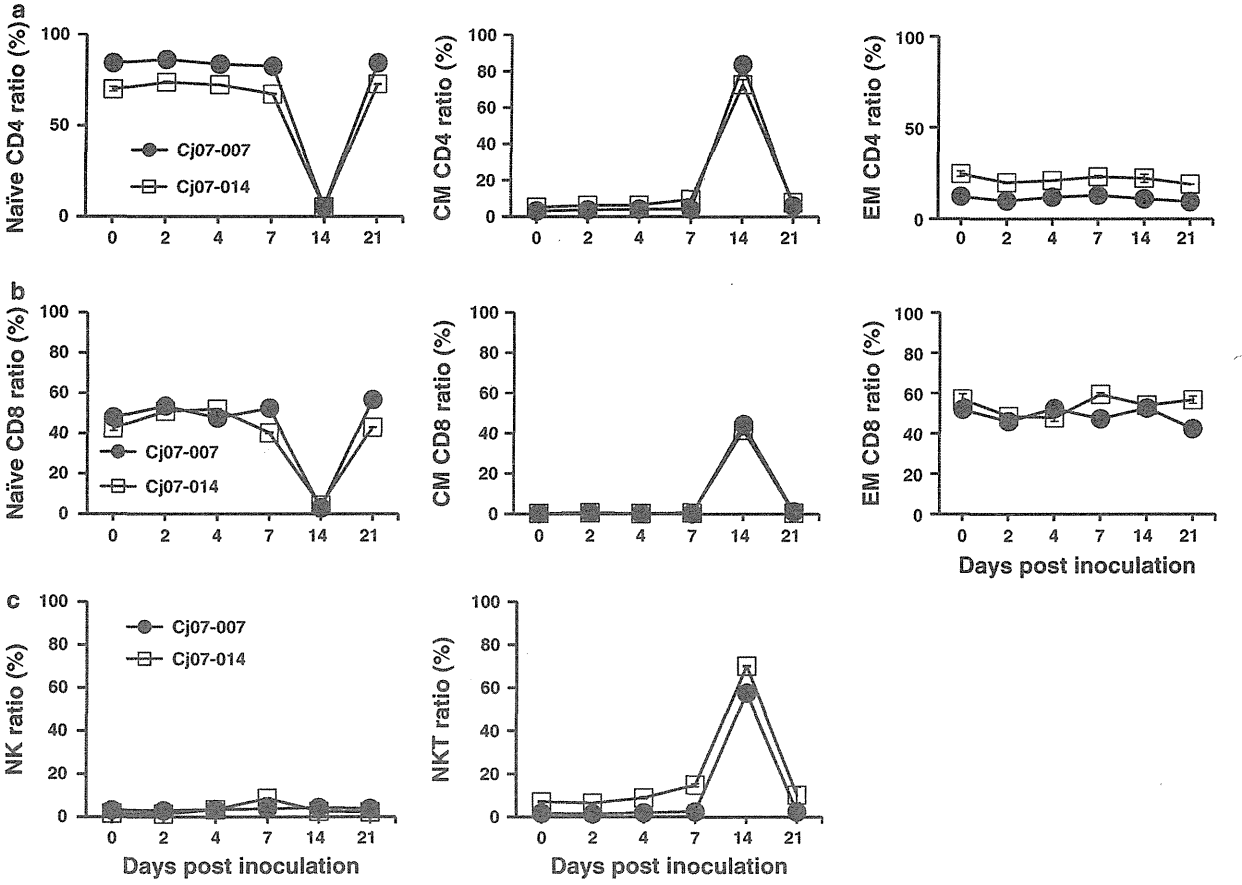

Fig. 7 Frequency of CD4 and CD8 T, NK and NKT cells in marmosets after re-challenge with the DENV-2 DHF0663 strain. Two marmosets initially inoculated with 1.8×10^5 PFU of the DHF0663 strain were re-inoculated 33 weeks after the primary challenge with 1.8×10^5 PFU of the same strain. (**a**) Ratios of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

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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

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

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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

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