

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- α / β R^{-/-} and IFN γ 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 γ 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- α / β R^{-/-} and IFN γ 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- α / β 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- α / β R^{-/-} and IFN γ R^{-/-} mice. Moreover, these mice were inoculated with 10⁹–10¹⁰ genome equivalents (GE) of DENV [27, 35, 36], which were likely in large excess compared with the 10⁴–10⁵ 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 × 10⁷ 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 (10⁵–10⁷ GE/ml) after subcutaneous inoculation with 10⁴–10⁵ 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 $27 \pm 2^\circ\text{C}$ in $50 \pm 10\%$ 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

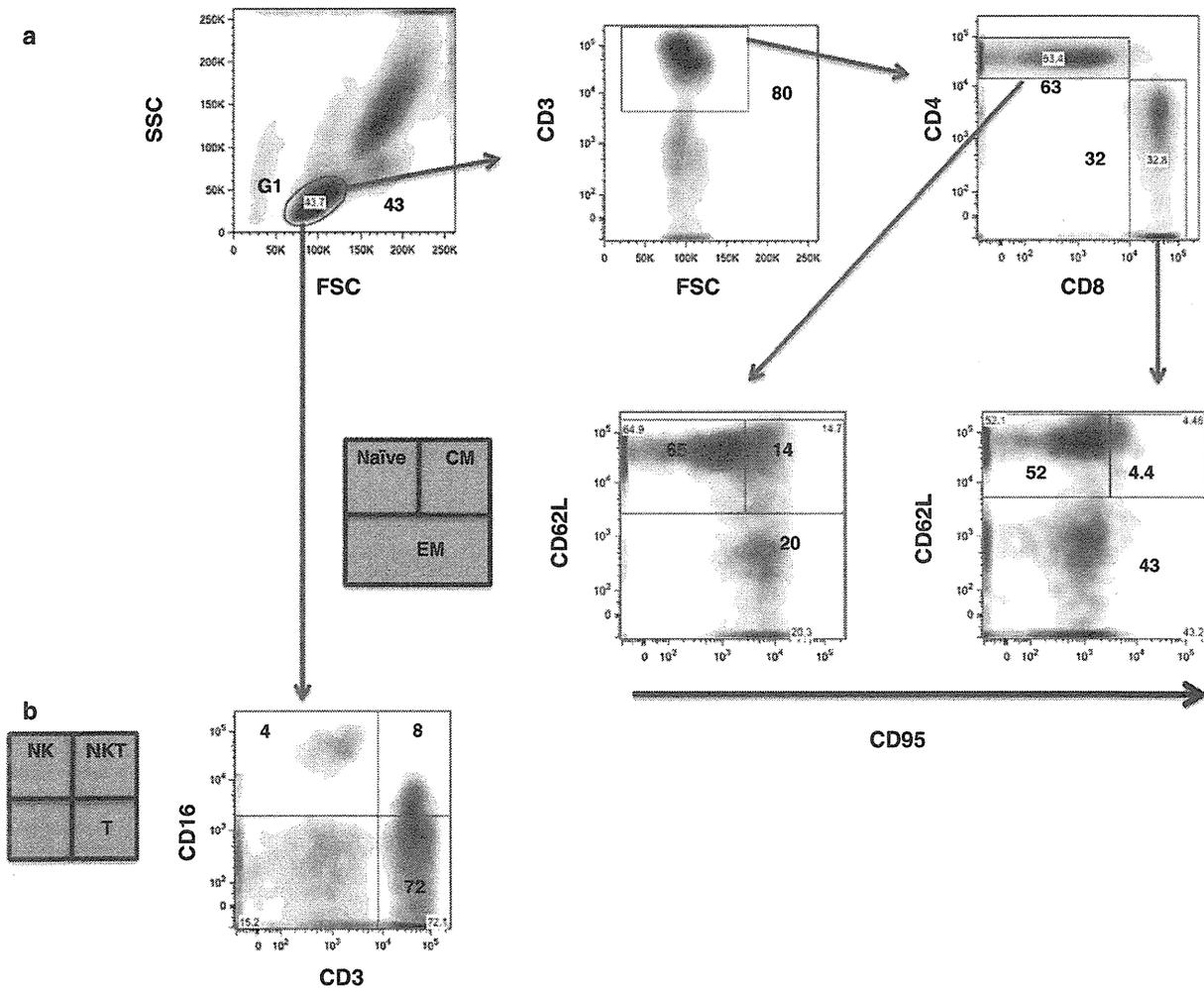


Fig. 1 Flow cytometric analysis of naïve, central/memory T cells and NK/NKT cells in marmosets. (a) Gating strategy to identify CD4 and CD8 T, NK and NKT cells. The G1 population was selected and analyzed for CD4 and CD8 T, NK and NKT cells.

(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 \pm standard deviation (SD) for the marmosets used in this study.

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⁻ naïve T cells (T_N), CD62L⁺CD95⁺ central memory T cells (T_{CM}), and CD62L⁻CD95[±] 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[±], 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

Table 1 Subpopulation ratios of lymphocytes in marmosets

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

SD: Standard deviation

Results shown are mean \pm SD from 3 healthy marmosets

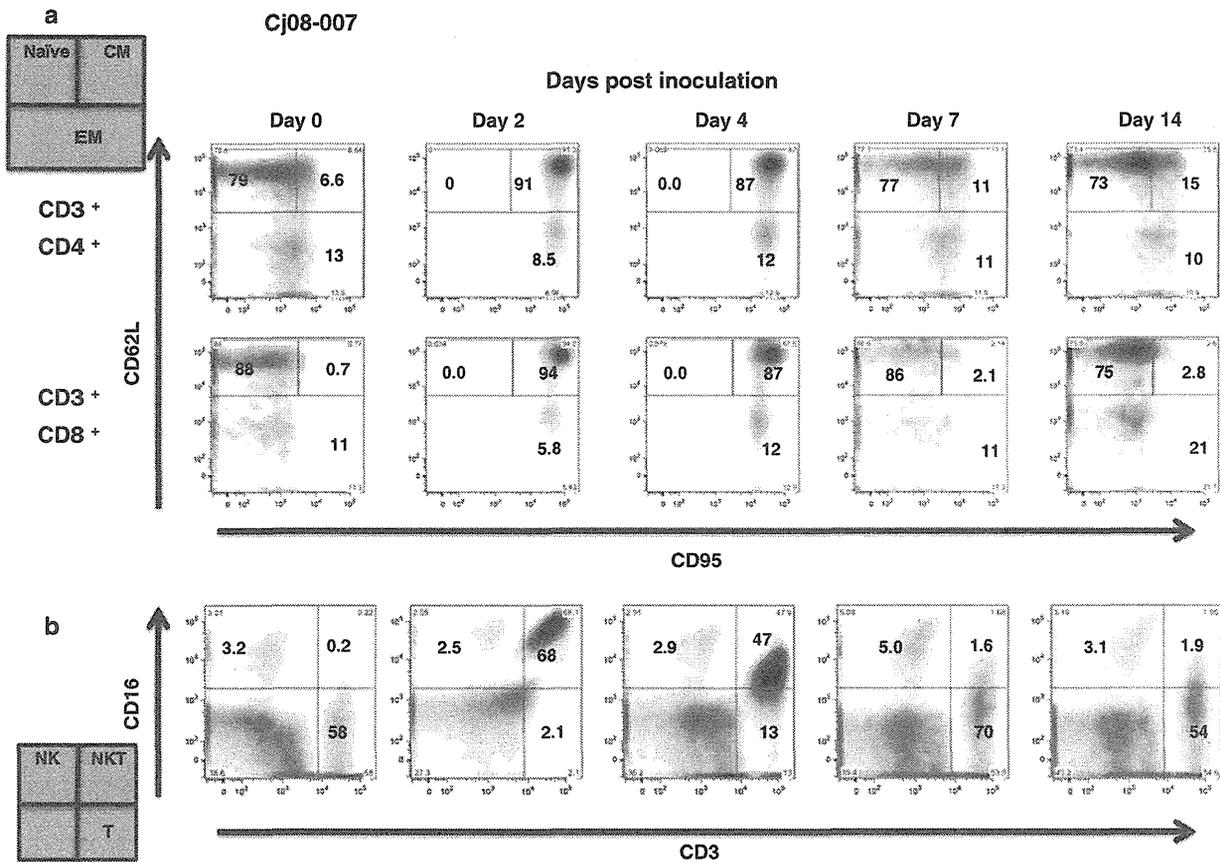


Fig. 2 Profiling of CD4 and CD8 T, NK and NKT cells in marmosets with primary infection with the DENV-2 Mal/77/08 strain. For primary DENV infection, two marmosets were inoculated subcutaneously in the back with 1.9×10^5 PFU of the DENV-2 Mal/

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.

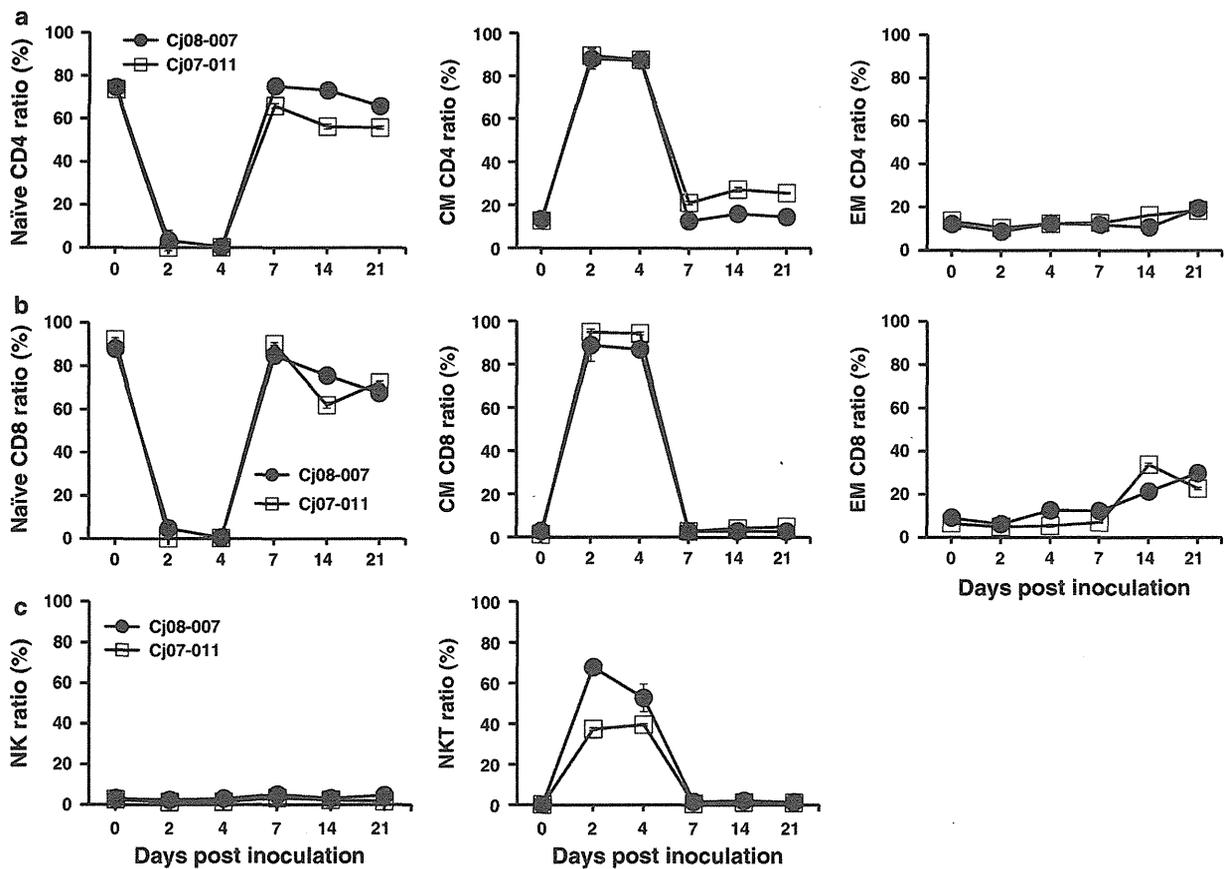


Fig. 3 Frequency of CD4 and CD8 T, NK and NKT cells in marmosets with primary infection with the DENV-2 Mal/77/08 strain. For primary DENV infection, two marmosets were inoculated subcutaneously in the back with 1.9×10^5 PFU of the DENV-2 Mal/77/08 strain. (a) Ratios of naïve, central memory, and effector 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 \pm SD: %)					
		Days after inoculation					
		Day 0	Day 2	Day 4	Day 7	Day 14	Day 21
CD3 ⁺ CD4 ⁺ CD62L ⁺ CD95 ^{hi}	(CD4 T _N)	74.1 \pm 0.9	1.6 \pm 3.3	0.2 \pm 0.3	70.5 \pm 5.5	64.8 \pm 9.7	60.8 \pm 5.9
CD3 ⁺ CD4 ⁺ CD62L ⁺ CD95 ⁺	(CD4 T _{CM})	13 \pm 0.4	88.7 \pm 2.8	87.4 \pm 0.2	16.8 \pm 5.0	21.6 \pm 6.5	20 \pm 6.4
CD3 ⁺ CD4 ⁺ CD62LCD95 [±]	(CD4 T _{EN})	12.8 \pm 0.9	9.5 \pm 1.0	12.3 \pm 0.4	12.3 \pm 0.5	134 \pm 3.2	189 \pm 1.4
CD3 ⁺ CD8 ⁺ CD62L ⁺ CD95 ^{hi}	(CD8 T _N)	89.9 \pm 2.5	2.5 \pm 4.7	0.3 \pm 0.3	87.5 \pm 3.3	68.7 \pm 79	69.8 \pm 3.1
CD3 ⁺ CD8 ⁺ CD62L ⁺ CD95 ⁺	(CD8 T _{CM})	2.1 \pm 0.8	91.9 \pm 5.5	90.6 \pm 4.2	2.8 \pm 0.5	3.5 \pm 08	3.8 \pm 1.2
CD3 ⁺ CD8 ⁺ CD62LCD95 [±]	(CD8 T _{EN})	7.8 \pm 1.6	5.6 \pm 0.8	9.0 \pm 4.1	9.5 \pm 3.1	27.6 \pm 72	26.3 \pm 4.3
CD3 ⁻ CD16 ⁺	(NK)	2.9 \pm 0.2	1.8 \pm 0.6	2.2 \pm 0.9	4.2 \pm 0.9	2.8 \pm 04	3.2 \pm 1.7
CD3 ⁺ CD16 ⁺	(NKT)	0.2 \pm 0.0	52.6 \pm 17	46.1 \pm 8.5	1.1 \pm 05	1.7 \pm 05	1.2 \pm 0.2

SD: Standard deviation

Results shown are mean \pm 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,

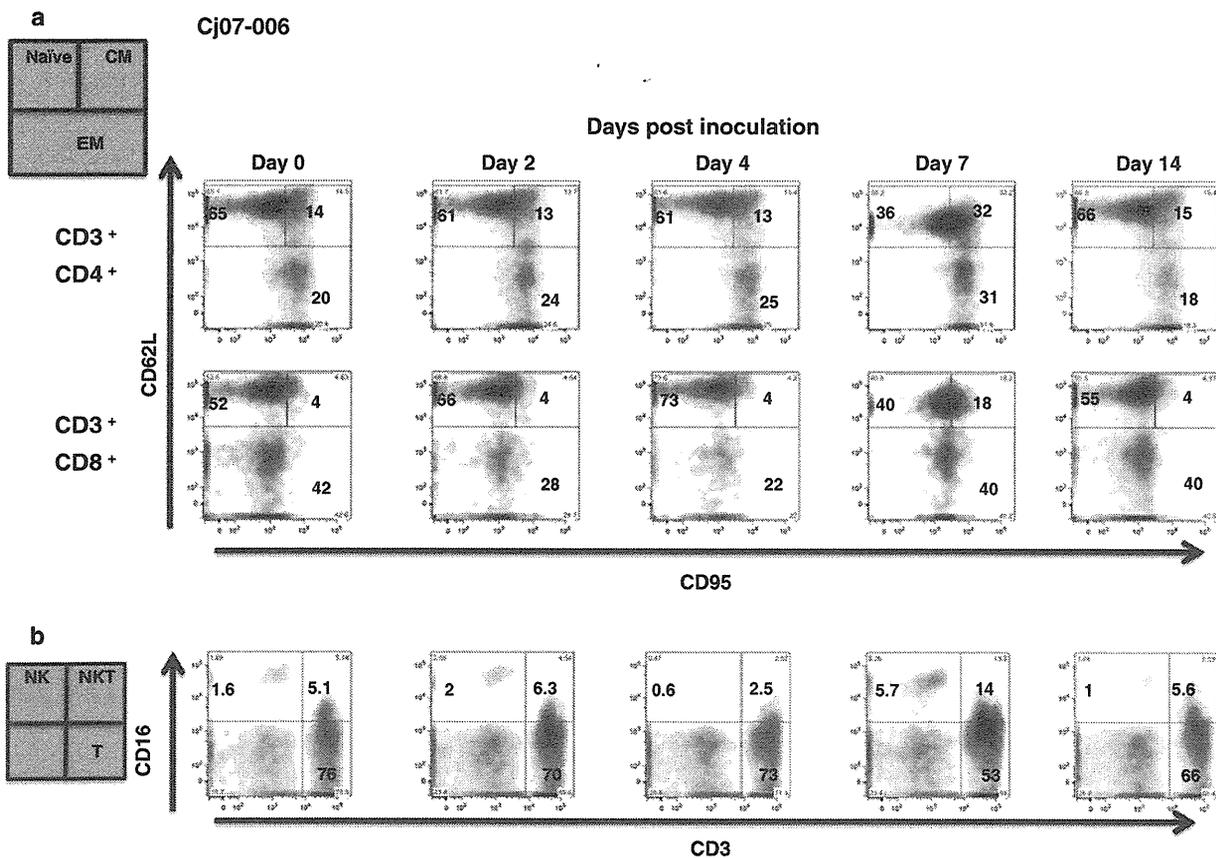


Fig. 4 Profiling of CD4 and CD8 T, NK and NKT cells in marmosets with primary infection with the DENV-2 DHF0663 strain. For primary DENV infection, two marmosets were inoculated subcutaneously in the back with 1.8×10^4 PFU of the DENV-2

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

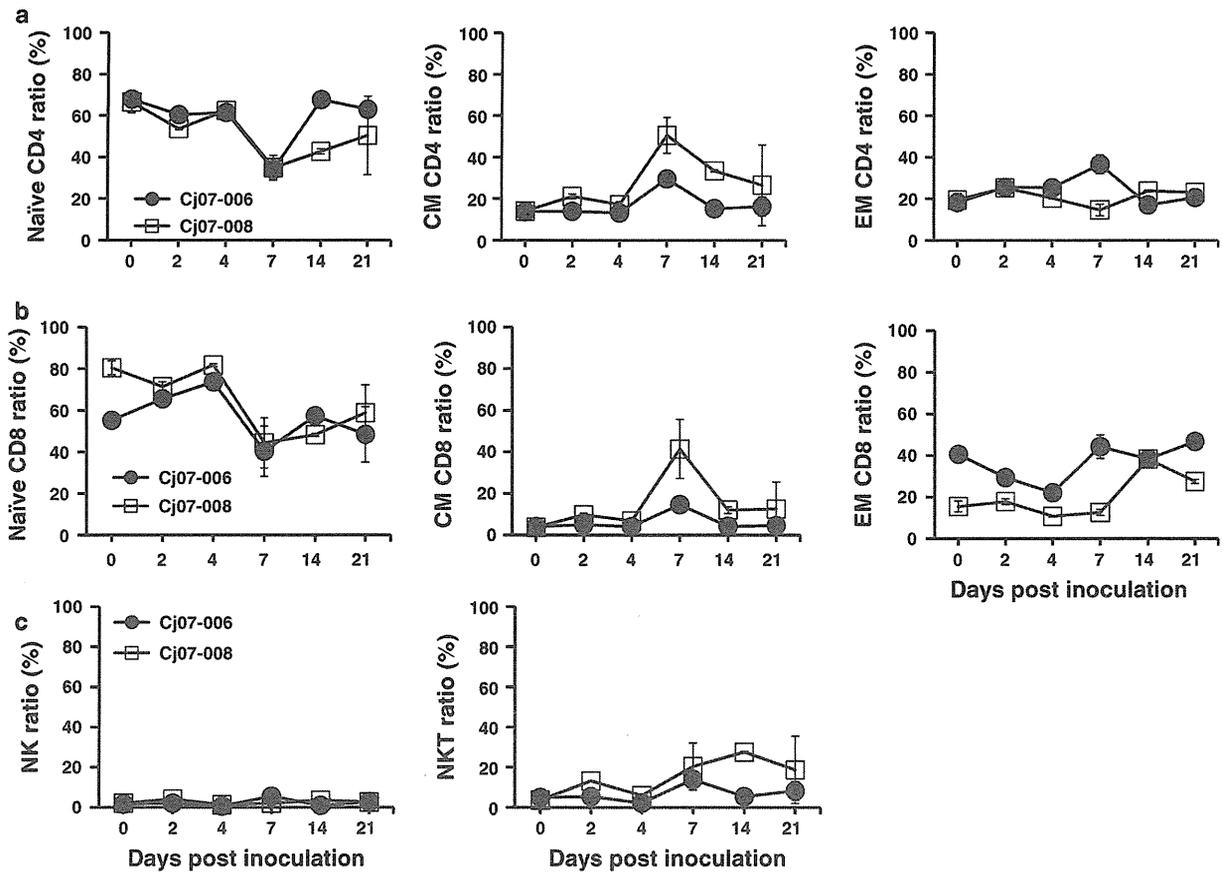


Fig. 5 Frequency of CD4 and CD8 T, NK and NKT cells in marmosets with primary infection with the DENV-2 DHF0663 strain. For primary DENV infection, two marmosets were inoculated subcutaneously in the back with 1.8×10^4 PFU of the DENV-2 DHF0663 strain. (a) Ratios of naïve, central memory, and effector

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

SD: Standard deviation

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

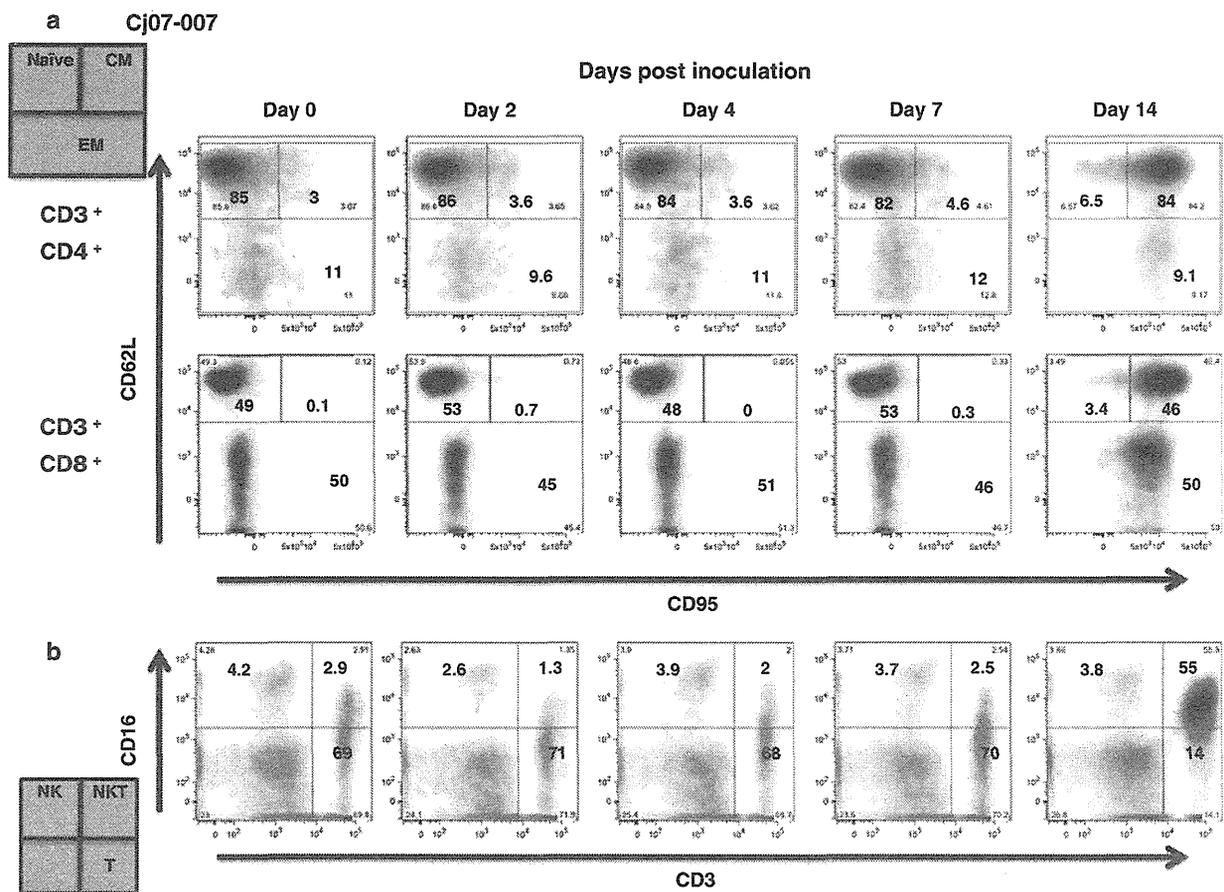


Fig. 6 Profiling of CD4 and CD8 T, NK and NKT cells in marmosets after re-challenge with the DENV-2 DHF0663 strain. Two marmosets that were initially inoculated with 1.8×10^5 PFU of the DHF0663 strain were re-inoculated 33 weeks after the primary

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

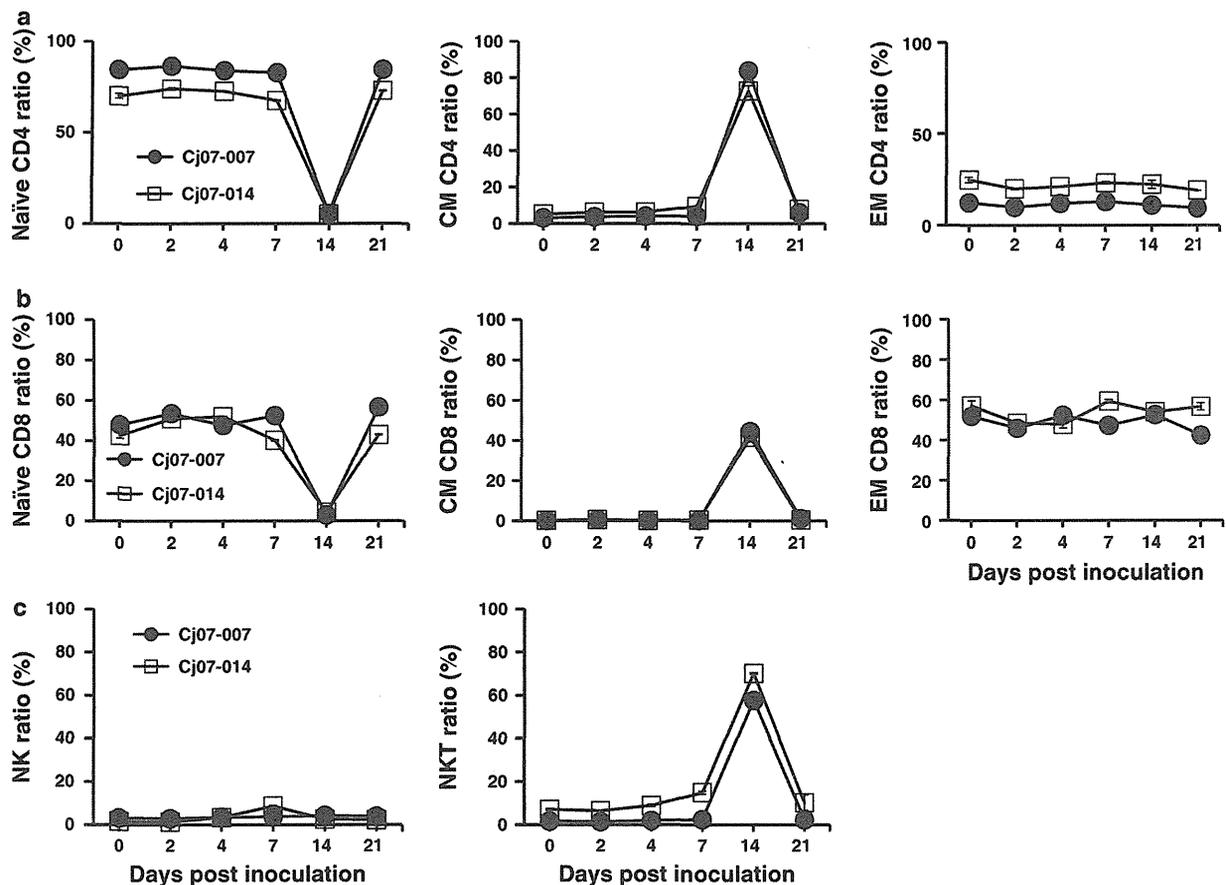


Fig. 7 Frequency of CD4 and CD8 T, NK and NKT cells in marmosets after re-challenge with the DENV-2 DHF0663 strain. Two marmosets initially inoculated with 1.8×10^5 PFU of the DHF0663 strain were re-inoculated 33 weeks after the primary challenge with 1.8×10^5 PFU of the same strain. (a) Ratios of naïve,

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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Single systemic administration of Ag85B of mycobacteria DNA inhibits allergic airway inflammation in a mouse model of asthma

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Abstract: The immune responses of T-helper (Th) and T-regulatory cells are thought to play a crucial role in the pathogenesis of allergic airway inflammation observed in asthma. The correction of immune response by these cells should be considered in the prevention and treatment of asthma. Native antigen 85B (Ag85B) of mycobacteria, which cross-reacts among mycobacteria species, may play an important biological role in host–pathogen interaction since it elicits various immune responses by activation of Th cells. The current study investigated the anti-allergic inflammatory effects of DNA administration of Ag85B from *Mycobacterium kansasii* in a mouse model of asthma. Immunization of BALB/c mice with alum-adsorbed ovalbumin followed by aspiration with aerosolized ovalbumin resulted in the development of allergic airway inflammation. Administration of Ag85B DNA before the aerosolized ovalbumin challenge protected the mice from subsequent induction of allergic airway inflammation. Serum and bronchoalveolar lavage immunoglobulin E levels, extent of eosinophil infiltration, and levels of Th2-type cytokines in Ag85B DNA-administered mice were significantly lower than those in control plasmid-immunized mice, and levels of Th1- and T-regulatory-type cytokines were enhanced by Ag85B administration. The results of this study provide evidence for the potential utility of Ag85B DNA inoculation as a novel approach for the treatment of asthma.

Keywords: immunotherapy, asthma, Ag85B, mycobacteria, allergy

Introduction

Asthma is characterized by airway hyperresponsiveness to a variety of specific and nonspecific stimuli, chronic pulmonary inflammation with eosinophilia, excessive mucus production, and high serum immunoglobulin E (IgE) levels. T-helper-2 (Th2) cells are thought to play a crucial role in the initiation, progression, and persistence of asthma in association with the production of interleukin-4 (IL-4), IL-5, and IL-13.^{1–3} Bronchoalveolar lavage (BAL) T-cells from human asthmatics have been reported to express elevated levels of IL-4 and IL-5 messenger ribonucleic acid (mRNA).^{4,5} Although the correction of this deviation to Th2-type immune responses is considered to be necessary to achieve therapeutic and preventive effects on asthma, it is not sufficient to obtain therapeutic effects in many cases. Another subset of T-cells, T-regulatory (Treg) cells, has been reported to be important in the development of allergic diseases such as asthma.⁶ Many studies have suggested that effective immunotherapy for allergic diseases is associated with immune deviation from a disease-promoting Th2 response towards a Th1 response, with Treg cells having appropriate functions.⁷ However, the induction of both subsets of cells – Th1 and Treg cells – for the treatment of asthma using immunological strategic tools is very difficult.

Administration of mycobacteria, including the bacillus Calmette–Guerin, has been thought to be effective for preventing the development of asthma by induction of Th1-type immune responses and inhibition of IgE by the production of IL-21 from natural killer T-cells.^{8–10} However, the relationship between bacillus Calmette–Guerin infection or mycobacteria immunization and asthma in humans is controversial because of the many causative factors affecting the induction of immune responses by mycobacteria, eg, human genetic background, mycobacteria strains, and environmental factors (reviewed in Arnoldussen et al).¹¹ From these findings, bacterial products from mycobacteria for immunotherapy against allergic disease should eliminate the harmful effects of host genetic factors, environmental factors, and strain specificity of mycobacteria.

Antigen 85B (Ag85B) is one of the most dominant protein antigens secreted from all mycobacterial species and has been shown to induce substantial Th cell proliferation and vigorous Th1 cytokine production.¹² Moreover, the induction of Th1-type immune responses by immunization of Ag85B was enhanced by presensitization with bacillus Calmette–Guerin.^{13,14} From these findings, the effectiveness of Ag85B DNA as immunotherapy for tumor disease and as a vaccine adjuvant for infectious disease, by its ability to induce Th1-type immune responses, was also reported.^{13,14} The current study investigated whether Ag85B DNA from *Mycobacterium kansasii* can inhibit the development of allergic airway inflammation as a novel immunotherapy.

Material and methods

Induction of allergic inflammation in mice

BALB/c female mice used in this study were handled according to ethical guidelines approved by the Institutional Animal Care and Use Committee of National Institute of Biomedical Innovation, Japan. The mice were sensitized to ovalbumin (OVA; Sigma-Aldrich, St Louis, MO) and challenged with aerosolized OVA according to a modification of the method of Nishikubo et al.¹⁵ Briefly, mice were subcutaneously immunized with 10 µg OVA complexed with alum on days zero and 14. On days 21–25 after the first immunization, mice were challenged with an aerosol of 5% OVA in phosphate-buffered saline in a chamber for 20 minutes.

Administration of DNA

Mice were intraperitoneally administered 50 µg plasmid DNA encoding Ag85B DNA once on day –7, zero, 14, or 21. An empty plasmid vector (pcDNA™ 3.1; Life Technologies, Carlsbad, CA) was used as a control (Figure 1A).

BAL fluid collection

BAL fluid was obtained by injecting and recovering two 0.5 mL aliquots of phosphate-buffered saline via a tracheal cannula. BAL fluid and sera were collected 25 days after the first OVA immunization. Cells in the BAL fluid were counted using a hemacytometer, and the differentials were determined by utilizing light microscopy to count 300 cells on Cytospin® preparations (Thermo Fisher Scientific, Waltham, MA). The concentration of inflammatory protein was measured by Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA).

Quantitation of IgE

IgE levels in sera were measured using enzyme-linked immunosorbent assay (ELISA) kits according to the procedure recommended by the manufacturer (Shibayagi Co, Ltd, Shibukawa, Japan).

Determination of cytokine production

Lymphocytes obtained from thoracic lymph nodes of immunized mice (5×10^6) were cultured with 10 µg/mL OVA in 24-well culture plates at a volume of 2 mL. After incubation at 37°C in a humidified incubator (5% carbon dioxide) for 48 hours, culture supernatants were collected and analyzed for production of interferon-γ (IFN-γ; Life Technologies) or IL-4 (Quantikine®, R&D Systems, Minneapolis, MN) by an ELISA assay according to the manufacturer's protocol (Life Technologies). The amounts of IL-5 and IL-13 in BAL fluid were also measured by an ELISA kit (R&D Systems) 25 days after the first OVA immunization.

Detection of cytokine mRNA from lymphocytes using real-time polymerase chain reaction

Total RNA was purified from OVA-stimulated or fetal calf serum (control)-stimulated spleen cells using Isogen (Nippon Gene Co, Ltd, Tokyo, Japan) following the manufacturer's instructions. For the real-time reaction, a reverse transcription system (Promega Corporation, Fitchburg, WI) was used. Polymerase chain reaction was performed in a total volume of 50 µL of 1 × polymerase chain reaction buffer (Takara Shuzo, Kyoto, Japan) containing 0.5–1.0 µg of complementary DNA, 0.25 mM of each deoxyribonucleotide triphosphate, 2 µM of each primer, and 2.5 U of *Taq* DNA polymerase (Takara Shuzo). The specific primer pairs used were described previously.¹⁵ The samples were amplified for 30–35 cycles under the following conditions: annealing for 30 seconds at 56°C, extension for 1 minute at 73°C, and denaturation for 30 seconds at 93°C. The reaction products were

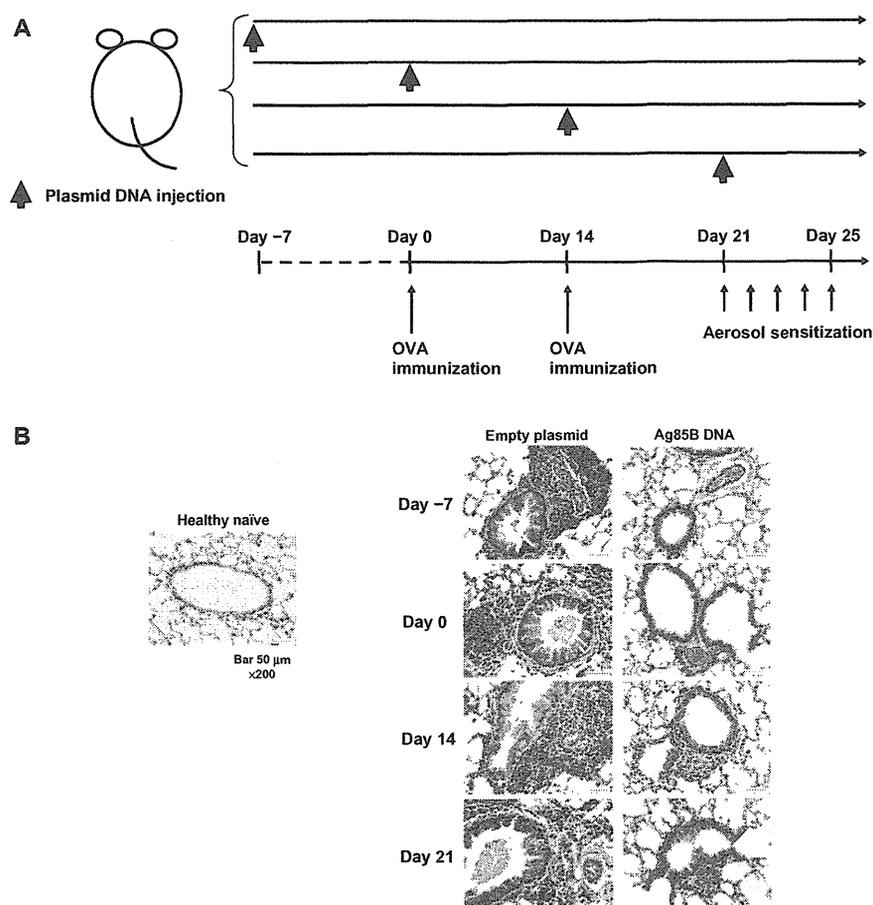


Figure 1 Inhibition of the development of allergic inflammation in lungs by administration of Ag85B DNA vaccine. **(A)** Experimental design used to investigate the effects of Ag85B DNA vaccine on OVA-induced asthma. Mice were subjected to an OVA sensitization scheme,¹⁵ and 50 µg of Ag85B DNA vaccine was intraperitoneally injected once on days -7, 0, 14, or 21. A control plasmid was also administered on the same day. **(B)** Results of histopathological examination of lungs of mice that had been administered Ag85B DNA or control DNA. All tissues were obtained 25 days after the first OVA immunization. The tissues were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Abbreviations: Ag85B, antigen 85B; OVA, ovalbumin.

analyzed on 2% agarose, Tris-buffered ethylenediaminetetraacetic acid gel. Photographs of the gels were scanned, and band intensities were measured using a densitometer (CS Analyzer 3.0; ATTO Corporation, Tokyo, Japan). The quantity of cytokine mRNA was determined by the ratio of cytokine and beta actin band intensities. The profiles shown are representative of three independent experiments.

Histopathological examinations

Histopathological examinations of the lungs of the mice that had been administered Ag85B DNA or control DNA were performed. All tissues were obtained 25 days after the first OVA immunization. The tissues were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Results for healthy naïve mice and control plasmid DNA-immunized mice are also shown.

Statistical analysis

Statistical analyses were performed using the Mann–Whitney U test and the Kruskal–Wallis test. Values are expressed as mean ± standard deviation. A 95% confidence limit was considered to be significant ($P < 0.05$).

Results

Inhibition of the development of allergic inflammation in the lung by administration of Ag85B DNA

Mice were sensitized to OVA and challenged with aerosolized OVA as described previously.¹¹ These mice were intraperitoneally administered 50 µg plasmid DNA encoding Ag85B once on day -7, zero, 14, or 21. An empty plasmid vector (pcDNA 3.1) was used as a control (Figure 1A).

Histopathological examinations of the lungs of mice injected with Ag85B DNA or control DNA and the lungs of healthy naïve mice were performed 25 days after the first inoculation of the plasmid. The lungs of mice that were administered Ag85B DNA on days -7, zero, and 14 did not show any pathological abnormalities compared with those of healthy naïve mice, but the lungs of mice that were administered Ag85B DNA on day 21 showed mild inflammation due to infiltration of eosinophils (Figure 1B). Mice administered the control plasmid did not show any inhibitory effects on the development of allergic inflammations. These results indicated that Ag85B DNA administration was effective for inhibiting the development of allergic inflammation, especially in the early phase of antigen sensitization.

Marked inhibition of allergic immune responses by administration of Ag85B DNA

The levels of protein, total cells, eosinophils, lymphocytes, and neutrophils in BAL fluid from mice immunized with Ag85B DNA vaccine were significantly lower than those in BAL fluid from mice vaccinated with control DNA (Figure 2A–F). Administration of Ag85B DNA also resulted in a significant reduction in the level of OVA-specific IgE (Figure 2G). The concentrations of Th2-type cytokines (IL-5 and IL-13) in BAL fluid from mice immunized with Ag85B DNA vaccine were significantly lower than those in BAL fluid from control mice (Figure 3A and B). These inhibitory effects on the development of allergic inflammation were correlated with day of Ag85B DNA injection. Injection on an early day was more effective for inhibiting the development of allergic inflammation. These results were also confirmed by histopathological observation.

Effects of Ag85B DNA administration on the production of IL-4 and IFN- γ in response to OVA

The production of OVA-specific cytokines in lymph node cells after *in vitro* stimulation with OVA were assessed. The lymphocytes obtained from thoracic lymph nodes were stimulated *in vitro* with OVA for 48 hours. IL-4 and IFN- γ levels were measured in culture supernatants by ELISA. The level of IL-4 in culture supernatants from cells of Ag85B DNA-immunized mice was much lower than in culture supernatants from cells of control mice (Figure 4A). On the other hand, the production level of IFN- γ in Ag85B DNA-immunized mice was significantly higher than in control DNA-immunized mice (Figure 4B).

Expression of cytokine mRNA in pulmonary lymph node cells after stimulation with OVA

The production of OVA-specific cytokines was also confirmed by mRNA levels of Th1-type cytokines (IFN- γ , IL-2, and IL-12) and Th2-type cytokines (IL-4, IL-5, and IL-13) (Figure 5A–C). Lymph node cells from Ag85B DNA vaccine-immunized mice showed strong IFN- γ , IL-2, and IL-12 expression and weak IL-4, IL-5, and IL-13 expression of mRNA, whereas control DNA-immunized mice showed the completely opposite results. The cells from control mice showed strong mRNA expression of Th2-type cytokines and weak mRNA expression of Th1-type cytokines (Figure 5A–C). It has been reported that therapeutic effects against asthma by administration of the culture supernatant of *M. vaccae* were derived from Treg cells by the induction of IL-10 and transforming growth factor- β .¹⁶ In the current study, mRNA expression levels of IL-10 and transforming growth factor- β in lymph node cells obtained from mice immunized with Ag85B DNA were much higher than those in lymph node cells obtained from control mice after *in vitro* stimulation with OVA (Figure 5A and D). Another Th17 cell lineage, which is associated with allergen-induced airway allergic inflammation, was also assessed by the mRNA expression of cytokines. In the current experiment, mRNA expression of IL-17 was seen in both control DNA-immunized and Ag85B-DNA immunized mice after stimulation with OVA, with no difference in the mRNA expression levels of IL-17 between these groups (Figure 5A and E). The mRNA expression of IL-23 was also assessed since IL-23 is associated with the maturation of Th17 cells.¹⁷ Expression of IL-23 mRNA was observed at the same level in all samples (Figure 5A and E). Inhibitory effects on the development of allergic inflammation are readily obtained in a mouse model of asthma through the administration of Ag85B DNA. These effects of immunotherapy by Ag85B DNA are due to activation of the immune responses of Th1 and Treg cells and inhibition of the responses of Th2 cells as a result of the enhancement of responses of Th1 and Treg cells.

Discussion

Current treatments of nonspecific immunosuppressive therapy for asthma, such as administration of glucocorticoids, are not satisfactory. Although these treatments are highly effective for controlling disease, most patients must continue to take these drugs throughout their lives. Moreover, these drugs have side effects, and asthma cannot be controlled by these drugs in up to 30% of patients. Given the high prevalence

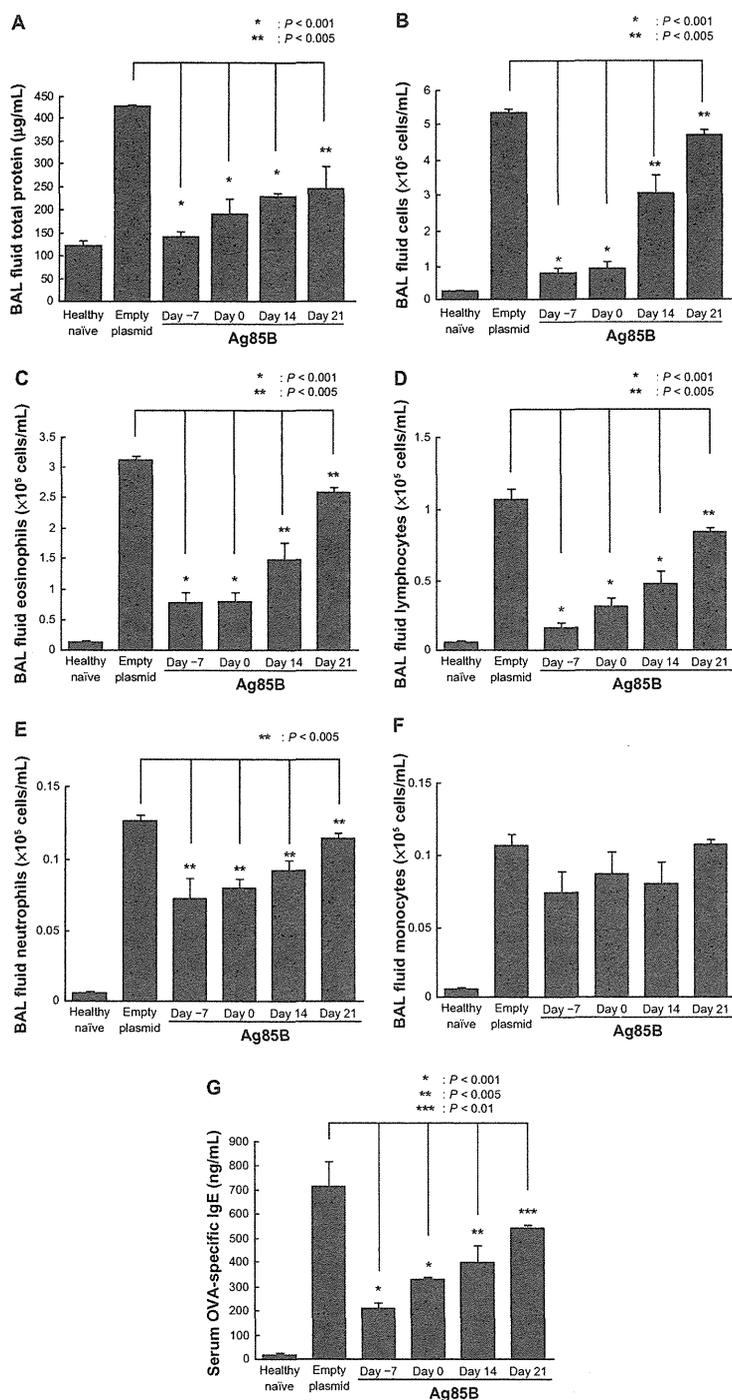


Figure 2 Marked inhibition of the development of allergic inflammation by administration of Ag85B DNA. BAL fluid was obtained by injecting and recovering two 0.5 mL aliquots of phosphate-buffered saline via a tracheal cannula. Cells in the lavage fluid were counted using a hemacytometer, and the differentials were determined by utilizing light microscopy to count 300 cells on Cytospin[®] preparations (Thermo Fisher Scientific, Waltham, MA). The concentration of inflammatory protein was measured by Protein Assay Reagent (Bio-Rad Laboratories, Hercules, CA). Results for healthy naive mice and control plasmid DNA-immunized mice are also shown. (A) Total protein, (B) number of cells, (C) eosinophils, (D) lymphocytes, (E) neutrophils, and (F) monocytes in BAL fluid from experimental animals were investigated. (G) The degrees of ovalbumin-specific immunoglobulin E responses in sera collected from experimental mice were also analyzed. Immunoglobulin E levels in sera were measured using enzyme-linked immunosorbent assay kits according to the procedure recommended by the manufacturer (Shibayagi Co, Ltd, Shibukawa, Japan). BAL fluid and sera were collected 25 days after the first ovalbumin immunization.

Notes: Data are representative of at least three independent experiments; values shown are the means and standard deviations of five mice per group; statistical analysis was performed using the Mann-Whitney U test and the Kruskal-Wallis test.

Abbreviations: Ag85B, antigen 85B; BAL, bronchoalveolar lavage.

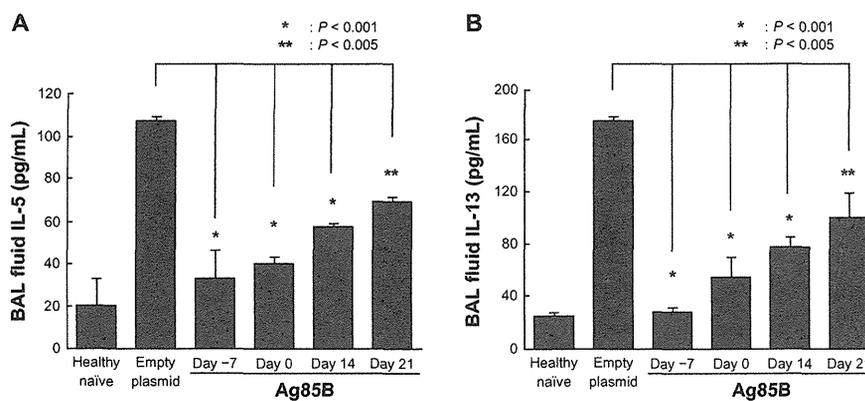


Figure 3 IL-5 and IL-13 production in BAL fluid. Amounts of (A) IL-5 and (B) IL-13 in BAL fluid were measured using an enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN) 25 days after the first ovalbumin immunization.

Notes: Data are representative of at least three independent experiments; values shown are means and standard deviations of five mice per group; statistical analysis was performed using the Mann-Whitney U test and the Kruskal-Wallis test.

Abbreviations: Ag85B, antigen 85B; BAL, bronchoalveolar lavage; IL, interleukin.

of this disease, improved and more effective therapeutic strategies are needed. The results of many studies have suggested that effective immunotherapy for allergic disease is associated with immune deviation from a disease-promoting Th2 response towards a Th1 response, with Treg cells having appropriate functions (reviewed in Takeda et al).¹⁸ In the current study, the applicability of plasmid encoding complementary DNA of Ag85B from mycobacteria DNA to gene therapy of asthma was assessed. Although the introduced DNA is expressed predominantly by somatic cells, it is known that a relatively small but biologically significant number of dendritic cells are transfected with the inoculated DNA.¹⁹⁻²¹ Moreover, it was recently reported that systemic inoculation of a plasmid DNA may cause dendritic cell

activation through direct transfection into dendritic cells.²² It was demonstrated that inhibitory effects on the development of allergic inflammation are readily obtained in a mouse model of asthma through the administration of Ag85B DNA, even with only a single administration before or after antigen sensitization.

The mechanism of immune responses induced by Ag85B remains unclear. Various products having adjuvant activities, eg, lipopolysaccharide, cytosine-phosphodiester-guanine motif, and polyinosinic:polycytidylic acid, involve toll-like receptors (TLRs) and show augmentation of Th1-type immune responses.¹⁸ It was previously reported that plasmid DNA encoding Ag85B stimulated the expression of TLR2, TLR3, and TLR4 mRNA. One possibility is that the induction

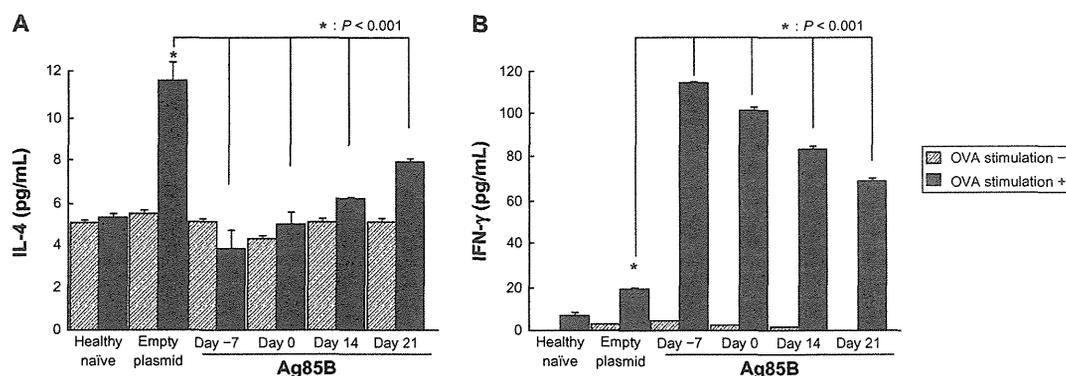


Figure 4 IFN-γ and IL-4 production in culture supernatant. Amounts of (A) IFN-γ and (B) IL-4 in culture supernatant were measured by enzyme-linked immunosorbent assay 25 days after the first OVA immunization. Spleen cells from immunized mice (5×10^6) were cultured with 10 μg/mL OVA in 24-well culture plates at a volume of 2 mL. After incubation at 37°C in a humidified incubator (5% carbon dioxide) for 96 hours, culture supernatants were quantified by using a standard enzyme-linked immunosorbent assay kit (Life Technologies, Carlsbad, CA).

Notes: Data are representative of at least three independent experiments; values represent mean and standard deviation of ten mice per group; statistical analysis was performed using the Mann-Whitney U test and the Kruskal-Wallis test.

Abbreviations: Ag85B, antigen 85B; IFN-γ, interferon-γ; IL-4, interleukin-4; OVA, ovalbumin.

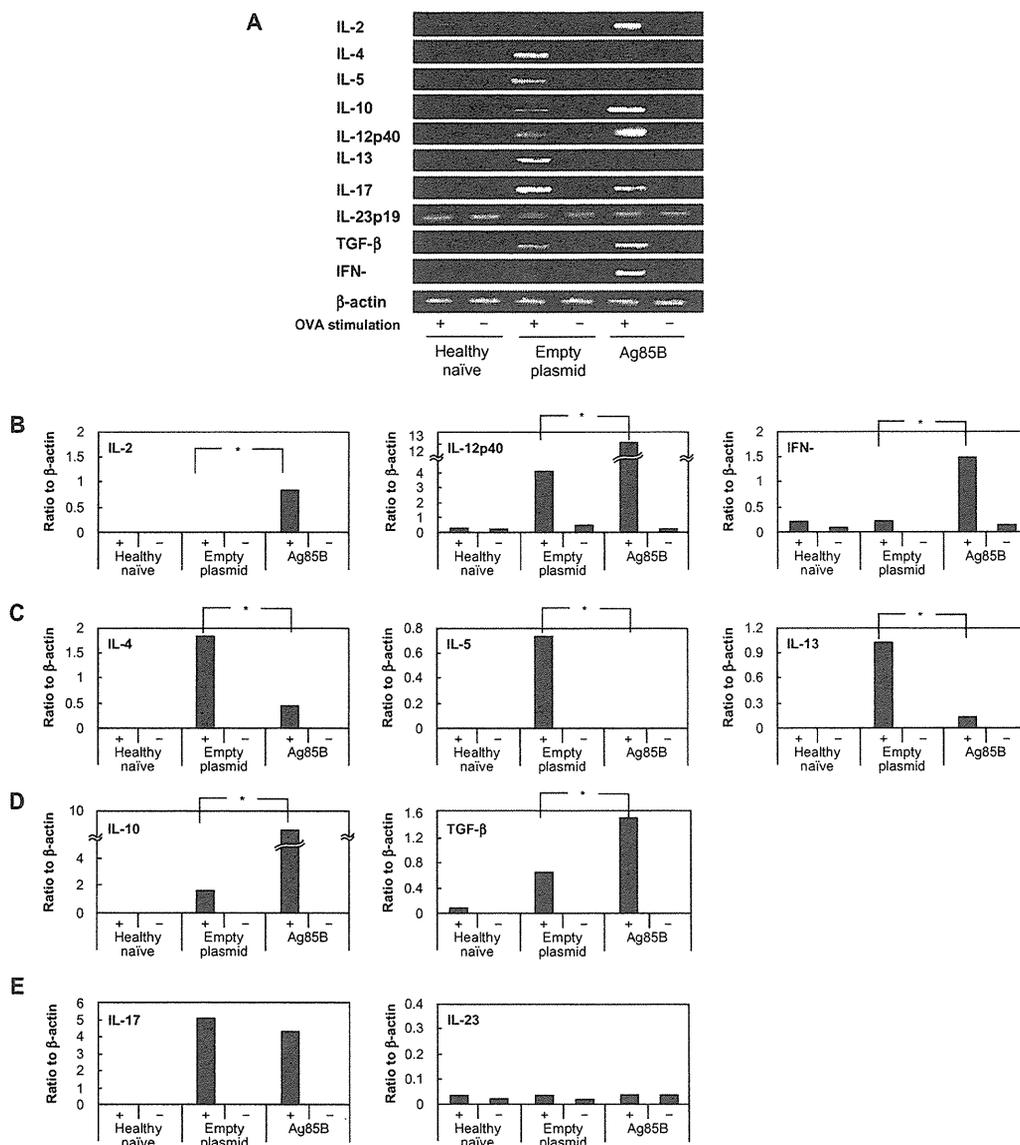


Figure 5 Detection of cytokine messenger ribonucleic acid from lymphocytes using real-time polymerase chain reaction. Spleen cells were stimulated in vitro with OVA for 1 day in culture. Spleen cells stimulated with fetal calf serum were used as controls. Total ribonucleic acid was purified from the OVA-stimulated or fetal calf serum (control)-stimulated spleen cells using Isogen (Nippon Gene Co, Ltd, Tokyo, Japan) following the manufacturer’s instructions. For the real-time reaction, a reverse transcription system (Promega Corporation, Fitchburg, WI) was used. Polymerase chain reaction was performed in a total volume of 50 μL of 1 × polymerase chain reaction buffer (Takara Shuzo, Kyoto, Japan) containing 0.5–1.0 μg of complementary DNA, 0.25 mM of each deoxyribonucleotide triphosphate, 2 μM of each primer, and 2.5 U of *Taq* DNA polymerase (Takara Shuzo). The specific primer pairs used were previously described.¹⁵ The samples were amplified for 30–35 cycles under the following conditions: annealing for 30 seconds at 56°C, extension for 1 minute at 73°C, and denaturation for 30 seconds at 93°C. (A) The reaction products were analyzed on 2% agarose, Tris-buffered ethylenediaminetetraacetic acid gels. (B–E) Photographs of the gels were scanned, and band intensities were measured using a densitometer (CS Analyzer 3.0; ATTO Corporation, Tokyo, Japan). The quantity of cytokine messenger ribonucleic acid was determined by the ratio of cytokine and beta actin band intensities.

Notes: **P* < 0.005; the profiles are representative of three independent experiments; statistical analysis was performed using the Mann–Whitney U test and the Kruskal–Wallis test.

Abbreviations: Ag85B, antigen 85B; IFN-γ, interferon-γ; IL, interleukin; OVA, ovalbumin; TGF-β, transforming growth factor-β.

of Th1-type immune responses by Ag85B is involved in innate immune responses. From this result, the activation of Th1 and Treg cells by Ag85B administration was thought to be involved in responses through stimulation of TLR2, TLR3, and TLR4, but not TLR9.¹⁴ Various proteins derived

from pathogens promote Th1 responses through stimulation of TLRs and subsequently through secretion of cytokines.¹⁸ It has also been reported that TLR signaling induces not only Th1-type immune responses but also secretion of various cytokines from Treg cells.^{23–27} Moreover, recent studies have

indicated that Th1 cells produce IL-10 as well as Th1-type cytokines by Notch regulation-dependent signal transducer and activator of transcription-4 signaling.²⁸ From these findings, effective immunotherapy by induction of both Th1 cell and Treg cell responses is thought to be possible by using appropriate materials. In fact, an asthma model of mice immunized with culture supernatant of mycobacteria, *M. vaccae*, showed Th1 and Treg responses.¹⁶ The results of the current study suggest that the administration of Ag85B DNA has several potential advantages due to the activation of Th1 and Treg cells for the prevention and treatment of asthma.

Immunization with mycobacteria or mycobacteria products has been reported to inhibit the development of allergic disease.^{29–32} However, various causative factors affect immune responses by mycobacteria. It was reported as a notable point that the efficacy of mycobacteria in preventing allergic inflammation of asthma was strongly affected by *Nramp1* alleles.³³ Several host genetic factors, including natural resistance-associated macrophage protein 1 (NRAMP1),³⁴ vitamin D receptor (VDR),^{35,36} and Mendelian susceptibility to mycobacterial disease,³⁷ have been reported to be involved in responses to mycobacteria (reviewed in Casanova and Abel).³⁸ Differences in immune responses induced by different mycobacteria strains have also been reported. The differential immune responses were mediated by lipid-extracted molecules of mycobacteria.³⁹ Moreover, environmental factors are important for immune responses induced by mycobacteria in therapy for atopic diseases.^{40,41} Presensitization of mycobacteria in the natural environment affects the induction of Th1-type immune responses by mycobacteria vaccination.^{9,42,43} However, the specific components of mycobacteria that inhibit the development of allergic responses have not been reported. Ag85B is a single component of mycobacteria, and this product might not be affected by various other mycobacteria factors involved in immune responses. In fact, Th1-type immune responses induced by Ag85B are not affected by *Nramp* in mice.^{44,45}

Wu et al demonstrated the effects of intranasal administration of Ag85B in a mouse model of asthma.⁴⁶ It was previously reported that Ag85B has strong adjuvant activities involving Th1 immune responses.¹⁴ Intranasal administration of a plasmid DNA (DNA vaccine) with adjuvant activities has been considered to be inappropriate for human use. Intranasal inactivated influenza vaccine, with adjuvant, induced Bell's palsy in humans. Therefore, intranasal inactivated influenza vaccine with adjuvant is no longer in clinical use.⁴⁷ Systemic administration of a plasmid DNA (DNA vaccine) is better than intranasal administration if the same effects of the plasmid

DNA can be induced. The current study demonstrated the usefulness of Ag85B DNA vaccine and provided evidence of the potential utility of Ag85B DNA vaccine for the prevention and treatment of asthma, even with only a single systemic administration before or after antigen sensitization.

Conclusion

The correction of immune response should be considered in the prevention and treatment of asthma. Ag85B has potential utility for the prevention and treatment of asthma even with only a single administration.

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Disclosure

The authors report no conflicts of interest in this work.

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