Original Article

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

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Abstract

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

Introduction

DENV causes the most prevalent arthropod-borne viral infections in the world [29]. Infection with one of the four serotypes of DENV will lead to dengue fever (DF) and sometimes the fatal dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) [12]. The serious diseases likely develop more frequently following secondary infection with a serotype of DENV different from that of the primary infection. Infection with DENV induces a high-titered neutralizing antibody 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, development of an effective tetravalent dengue vaccine is considered to be of public health priority. There are recently several vaccine candidates for DENV infection under clinical trials, and most of them target the induction of neutralizing antibodies [20].

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 post-infection [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 detail regarding the implication of humoral and cellular immunity in controlling DENV infection remains 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 lymphocytes (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 DENV-2 lethal 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 protecting 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 that 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, CTL are also proposed to contribute to clearance during primary DENV infection and in pathogenesis during secondary heterologous infection in the BALB/c mouse model [4].

Why did the mouse models in 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 the 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 large excess as compared with humans injected with 10^4-10^5 GE of DENV by a mosquito [19]. In addition, efficiency of DENV replication in wild mice *in vivo* was very low compared with 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 ($1x10^7$ 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 unit (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 as 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 dynamics of adaptive immune responses *in vivo*.

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

Materials and methods

Animals

All animal studies were conducted in accordance with the 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 6 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 12h 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 a healthy condition and confirmed to be negative for anti-dengue virus antibodies before inoculation with dengue virus [24].

Cells

Cell culture was performed as previously described [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), DHF0663 strain (Accession no. AB189122) and D2/Hu/Maldives/77/2008NIID (Mal/77/08) strain 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 Maldives. All DENV strains isolated clinical samples were propagated with C6/36 cells and were used within 4 passages on C6/36 cells. Culture supernatant from infected C6/36 cells was 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, the profiling of the key adaptive and innate immune cells in the marmosets after infection with serotype 2 of DENV (DENV-2) was examined. At the primary DENV infection, four marmosets were inoculated subcutaneously in the back with either 1.9 $x10^5$ PFU of the DENV-2 Mal/77/08 strain (Cj08-007, Cj07-011) or $1.8x10^4$ PFU of the DHF0663 strain (Cj07-006, Cj07-008) [24]. In the case of the DENV re-challenge experiment, two marmosets initially inoculated with $1.8x10^5$ PFU of the DHF0663 strain were re-inoculated 33 weeks after the primary challenge with $1.8x10^5$ 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 was 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 were shown in Supplementary Figure 1 [24].

Flow cytometry

Flow cytometry was performed as previously described [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% fetal calf serum (FCS), the labeled cells were resuspended in a fix buffer containing PBS and 1% formaldehyde. The expression of these markers on the lymphocytes was analyzed with FACSCanto II flow cytometer (Becton Dickinson). The data analysis was conducted using a FlowJo software (Treestar, Inc.). Results were shown as mean±standard deviation (SD) from 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 Figure 1a. Human T cells are classically divided into 3 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 3 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, so far 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 previously reported [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 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 the marmosets infected with primary 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 post-infection (Supplementary Fig. 1a). For each of the two marmosets (Cj08-007, Cj07-011), the plasma levels of vRNA reached their peaks at $9.6x10^6$ and $7.0x10^6$ GE/ml on day 4 post-infection, respectively. The plasma vRNA was detected in both marmosets on days 2, 4, and 7. We then examined the profiling 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 completely 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 periods. $CD8^+$ 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 periods. 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 infected with primary DENV-2 (DHF0663 strain)

Next, we investigated the cellular immune responses against another DENV-2 DHF strain (DHF0663) in marmosets. Dengue vRNA was detected in plasma samples from the marmosets on day 2 post-infection ([24], Supplementary Fig. 1b). For each of the two marmosets (Cj07-006, Cj07-008), the plasma vRNA levels were shown to be $3.4x10^5$ and $3.8x10^5$ GE/ml on day 2 and $2.0x10^6$ and $9.4x10^5$ GE/ml at the peak on day 4 post-infection, respectively, followed by being undetectable on day 14. Thus, we examined the profiling 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 than 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 the marmosets re-challenged with a DENV-2 strain

In order to examine the cellular immune responses against the re-challenge of DENV-2 DHF strain in marmoset model, marmosets were infected twice with the same DENV-2 strain (DHF0663) at 33 weeks interval 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 to the re-challenge of the same DENV strain ([24]; Supplementary Fig.1c). We also investigated the profiling of the CD4 and CD8 T, NK and NKT cells in the marmosets (Cj07-007, Cj07-014) re-challenged with the same DENV-2 strain (DHF0663) (Fig. 6-7). $CD4^+$ T_{CM} cells drastically increased on day 14 post-inoculation. On the other hand, $CD4^+$ T_N cells completely decreased at the same time. $CD4^+$ T_{EM} cells maintained their initial levels through the observation periods. 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 were likely to be dependent on the strain of the virus employed for challenge experiments (Fig. 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, irrespective of the complete inhibition of the DENV replication. (Fig. 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 that of 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 day14 after the re-challenge) responses of CD4/CD8 central memory T cells were observed irrespective of the complete inhibition of the DENV replication. These results indicate that the cellular immunity is unlikely to play a major role in the control of the DENV re-infection. Alternatively, it is still possible that 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 may influence the strength of cellular immune responses. The differences in cellular immune responses between the monkeys infected with the DF or DHF strain may not be caused by individual differences in marmosets because the FACS results were consistent with each 2 marmosets. It was previously shown that there was a reduction in CD3, CD4, and CD8 cells in DHF and demonstrated 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 the IFN-inducible genes, IFN-induced genes and IFN production were strongly up-regulated in the DF patients compared with the DHF patients, suggesting a significant role of IFN system during DF strain infection compared with DHF strain infection [34]. Thus, it is reasonable to assume that the DHF strain might have an ability to negatively regulate T cell responses. A recent report demonstrating that the sequence of the DHF strain differed from that of DF strain in six unique amino acid residues located in the membrane, envelope and non-structural genes [33], which supports our notion.

Alternatively, the other possibility is that the strength of T cell responses might depend on the viral loads. In fact, in our results the greater T cell responses in the DF strain-infected monkeys were paralleled with higher viral loads, which was in contrast with the result of the DHF strain-infected animals with lower viral loads. Of note, the ten-fold more challenge dose of the DF strain used in this study (1.9×10^5 PFU) than that of the DHF strain (1.8×10^4 PFU) could have simply led to ten-fold more peak viral RNA levels in the DF strain-infected monkeys. In either case, the relationship between the strength of antiviral immune responses and the viral strains remains to be elucidated. Further *in vivo* characterization of the antiviral immunity and the viral replication kinetics induced by infection of various DENV strains isolated from DF and DHF patients will help 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 at 33 weeks as 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 result. 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 cooperatively acted 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 the immunological study in the course of the infection, which is likely the reason for the discrepancy regarding the importance of cellular immunity in DENV infection. In this point of view, our marmoset model of DENV infection will further provide important information regarding the roles of cellular immune responses in DENV infection.

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364 **Figure legends**

365 **Fig.1 Flow cytometric analysis of naïve, central/effector memory T cells and**
366 **NK/NKT cells in marmosets.** (a) Gating strategy to indentify the CD4 and CD8 T, NK
367 and NKT cells. The G1 population was selected and analyzed for CD4 and CD8 T, NK
368 and NKT cells. (a) Profiling of naïve, central memory, and effector memory CD4 and
369 CD8 T cells in total CD4 and CD8 T cells. (b) Profiling of NK and NKT cells in total
370 lymphocytes. Results shown are representative of 3 healthy marmosets used in this
371 study.
372

373 **Fig. 2 Profiling of CD4 and CD8 T, NK and NKT cells in marmosets with primary**
374 **infection of DENV-2 Mal/77/08 strain.** At the primary DENV infection, two
375 marmosets were inoculated subcutaneously in the back with 1.9 $x10^5$ PFU of the
376 DENV-2 Mal/77/08 strain. (a) Profiling of naïve, central memory, and effector memory
377 CD4 and CD8 T cells in total CD4 and CD8 T cells. (b) Profiling of NK and NKT cells
378 in total lymphocytes. (a-b) Cj08-007.
379

380 **Fig. 3 Frequency of CD4 and CD8 T, NK and NKT cells in marmosets with**
381 **primary infection of DENV-2 Mal/77/08 strain.** At the primary DENV infection, two
382 marmosets were inoculated subcutaneously in the back with 1.9 $x10^5$ PFU of the
383 DENV-2 Mal/77/08 strain. (a) Ratios of naïve, central memory, and effector memory
384 CD4 T cells in total CD4 T cells. (b) Ratios of naïve, central memory, and effector
385 memory CD8 T cells in total CD8 T cells. (c) Ratios of NK and NKT cells in total
386 lymphocytes. (a-c) Cj08-007, Cj07-011.
387

388 **Fig. 4 Profiling of CD4 and CD8 T, NK and NKT cells in marmosets with primary**
389 **infection of DENV-2 DHF0663 strain.** At the primary DENV infection, two
390 marmosets were inoculated subcutaneously in the back with 1.8$x10^4$ PFU of the
391 DENV-2 DHF0663 strain. (a) Profiling of naïve, central memory, and effector memory
392 CD4 and CD8 T cells in total CD4 and CD8 T cells. (b) Profiling of NK and NKT cells
393 in total lymphocytes. (a-b) Cj07-006.
394

395 **Fig. 5 Frequency of CD4 and CD8 T, NK and NKT cells in marmosets with**
396 **primary infection of DENV-2 DHF0663 strain.** At the primary DENV infection, two

397 marmosets were inoculated subcutaneously in the back with $1.8x10^4$ PFU of the
398 DENV-2 DHF0663 strain. (a) Ratios of naïve, central memory, and effector memory
399 CD4 T cells in total CD4 T cells. (b) Ratios of naïve, central memory, and effector
400 memory CD8 T cells in total CD8 T cells. (c) Ratios of NK and NKT cells in total
401 lymphocytes. (a-c) Cj07-006, Cj07-008.
402
403 **Fig. 6 Profiling of CD4 and CD8 T, NK and NKT cells in marmosets with**
404 **re-challenging DENV-2 DHF0663 strain.** In the case of the DENV re-challenge study,
405 two marmosets initially inoculated with $1.8x10^5$ PFU of the DHF0663 strain were
406 re-inoculated 33 weeks after the primary challenge with $1.8x10^5$ PFU of the same strain.
407 (a) Profiling of naïve, central memory, and effector memory CD4 and CD8 T cells in
408 total CD4 and CD8 T cells. (b) Profiling of NK and NKT cells in total lymphocytes.
409 (a-b) Cj07-007.
410
411 **Fig. 7 Frequency of CD4 and CD8 T, NK and NKT cells in marmosets with**
412 **re-challenging DENV-2 DHF0663 strain.** In the case of the DENV re-challenge study,
413 two marmosets initially inoculated with $1.8x10^5$ PFU of the DHF0663 strain were
414 re-inoculated 33 weeks after the primary challenge with $1.8x10^5$ PFU of the same strain.
415 (a) Ratios of naïve, central memory, and effector memory CD4 T cells in total CD4 T
416 cells. (b) Ratios of naïve, central memory, and effector memory CD8 T cells in total
417 CD8 T cells. (c) Ratios of NK and NKT cells in total lymphocytes. (a-c) Cj07-007,
418 Cj07-014.

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Conflict of Interest Statement:

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.

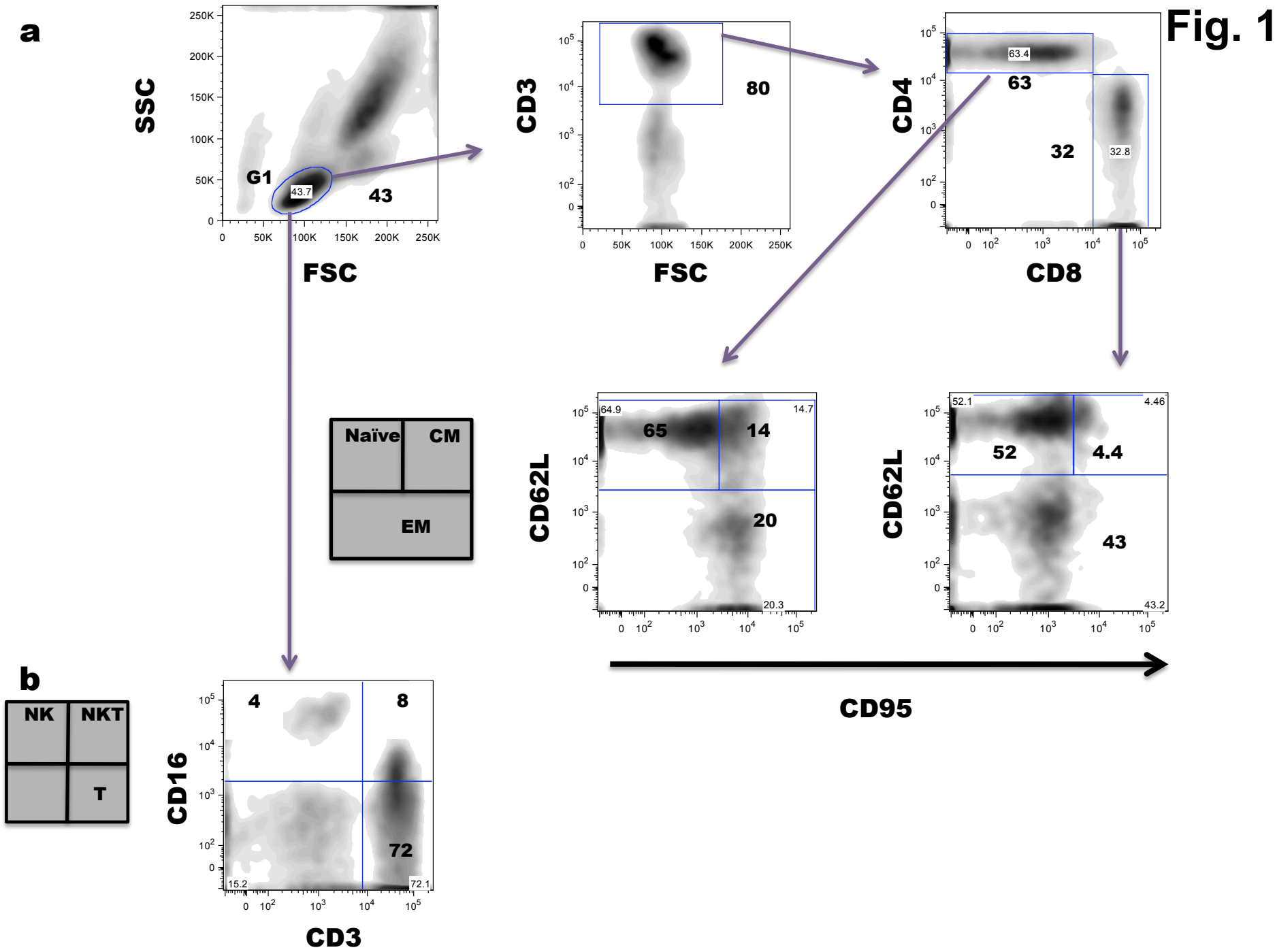
a
SSC
FSC
G1
43.7
43
CD3
80
FSC
CD4
63.4
63
32
32.8
CD8
Naïve
CM
EM
CD62L
64.9
65
14.7
14
20
20.3
CD62L
52.1
52
4.46
4.4
43
43.2
CD95
b
NK
NKT
T
CD16
4
8
72
15.2
72.1
CD3

Fig. 1

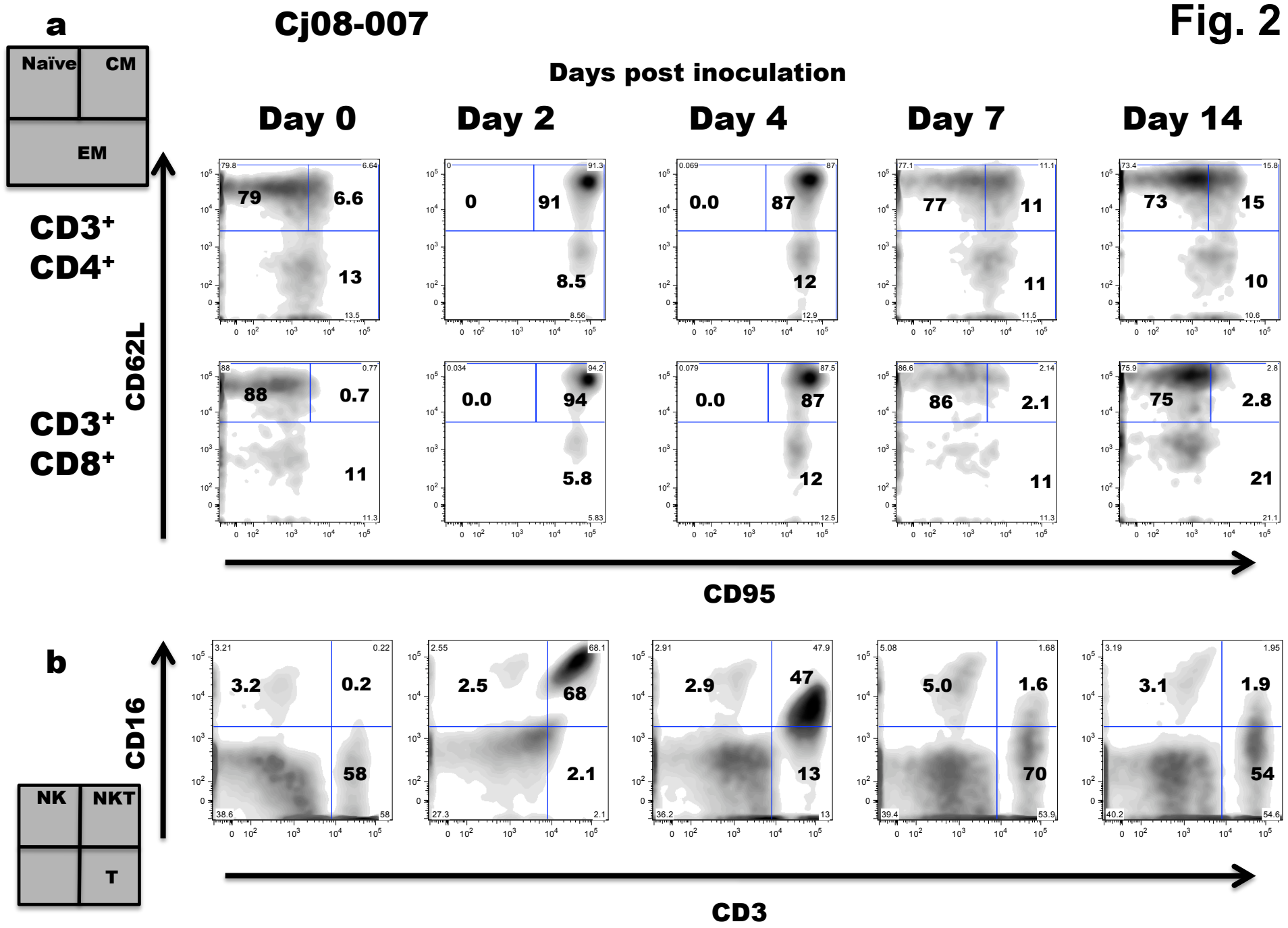
a
Cj08-007
Fig. 2
Naïve
CM
EM
Days post inoculation
Day 0
Day 2
Day 4
Day 7
Day 14
CD3+ CD4+
CD3+ CD8+
CD62L
79
6.6
13
0
91
8.5
0.0
87
12
77
11
11
73
15
10
88
0.7
11
0.0
94
5.8
0.0
87
12
86
2.1
11
75
2.8
21
CD95
b
CD16
NK
NKT
T
3.2
0.2
58
2.5
68
2.1
2.9
47
13
5.0
1.6
70
3.1
1.9
54
CD3

Fig. 3

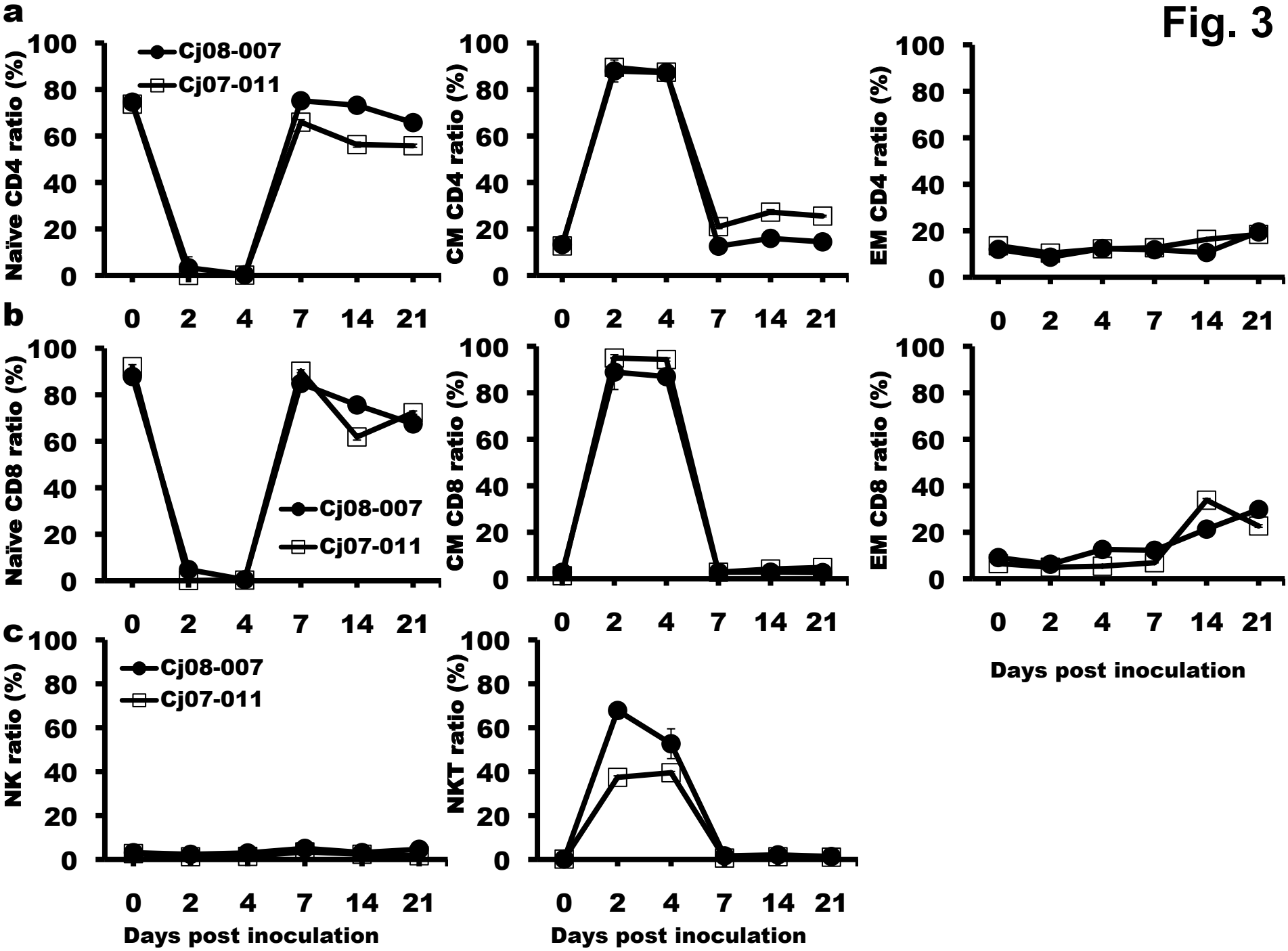
a
Naïve CD4 ratio (%)
Cj08-007
Cj07-011
CM CD4 ratio (%)
EM CD4 ratio (%)
b
Naïve CD8 ratio (%)
Cj08-007
Cj07-011
CM CD8 ratio (%)
EM CD8 ratio (%)
c
NK ratio (%)
Cj08-007
Cj07-011
NKT ratio (%)
0 2 4 7 14 21
Days post inoculation
Days post inoculation

Fig. 4

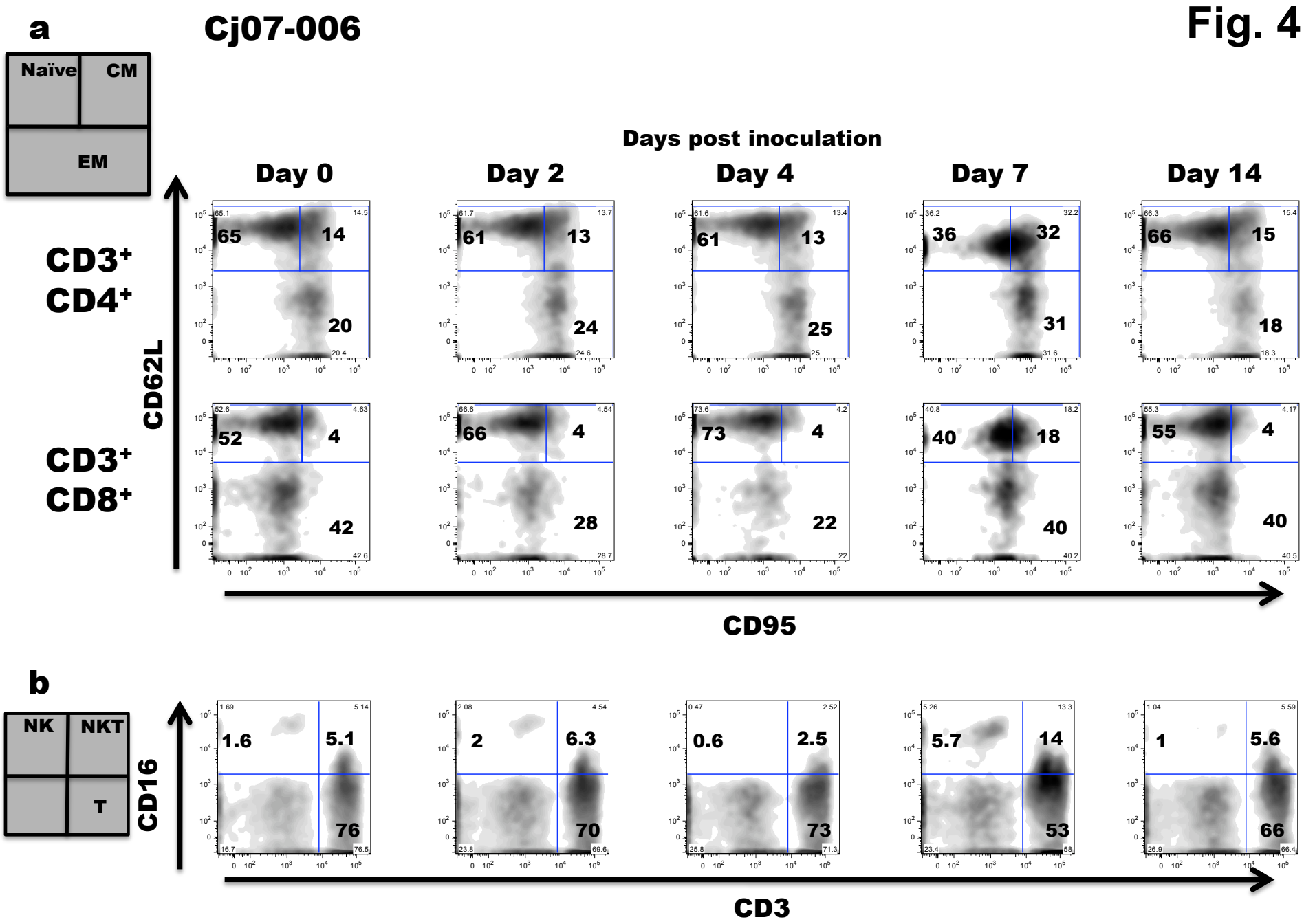
a
Cj07-006
Naïve
CM
EM
Days post inoculation
Day 0
Day 2
Day 4
Day 7
Day 14
CD3+ CD4+
CD3+ CD8+
CD62L
CD95
65
14
20
61
13
24
61
13
25
36
32
31
66
15
18
52
4
42
66
4
28
73
4
22
40
18
40
55
4
40
b
NK
NKT
T
CD16
CD3
1.6
5.1
76
2
6.3
70
0.6
2.5
73
5.7
14
53
1
5.6
66

Fig. 5

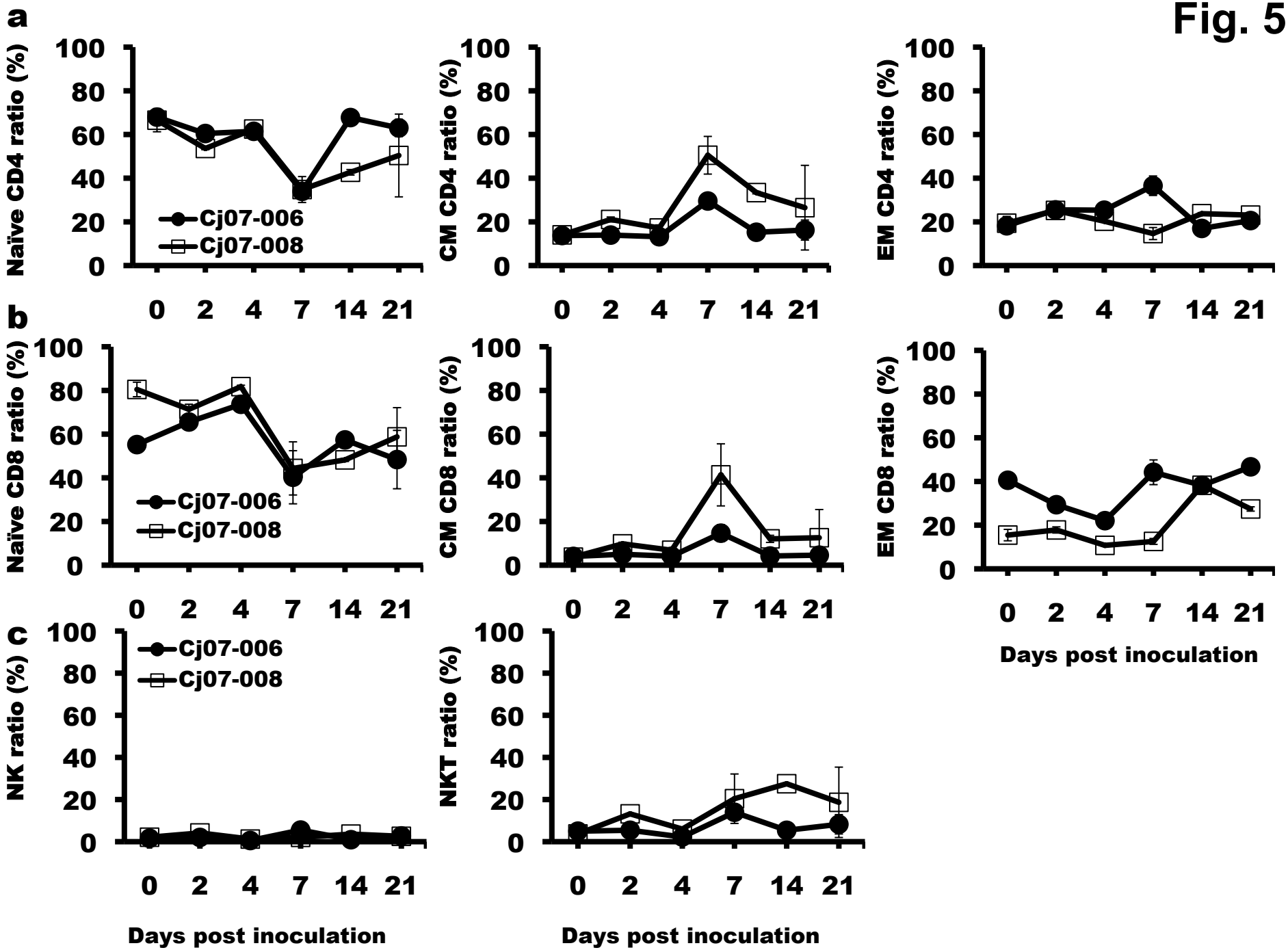
a
Naïve CD4 ratio (%)
CM CD4 ratio (%)
EM CD4 ratio (%)
b
Naïve CD8 ratio (%)
CM CD8 ratio (%)
EM CD8 ratio (%)
c
NK ratio (%)
NKT ratio (%)
Cj07-006
Cj07-008
0
20
40
60
80
100
0 2 4 7 14 21
Days post inoculation

Fig. 6

a Cj07-007

Days post inoculation

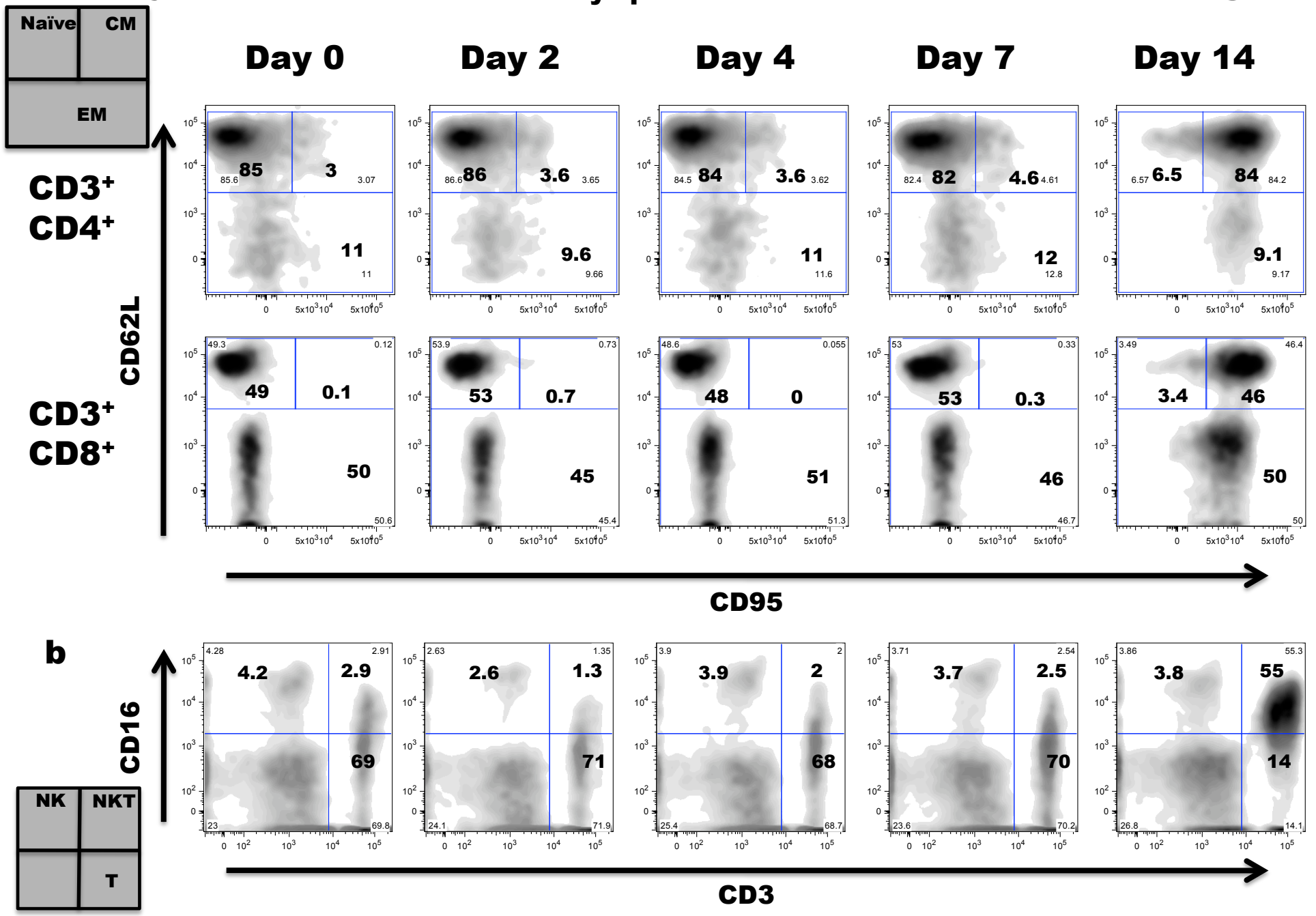

Fig. 7

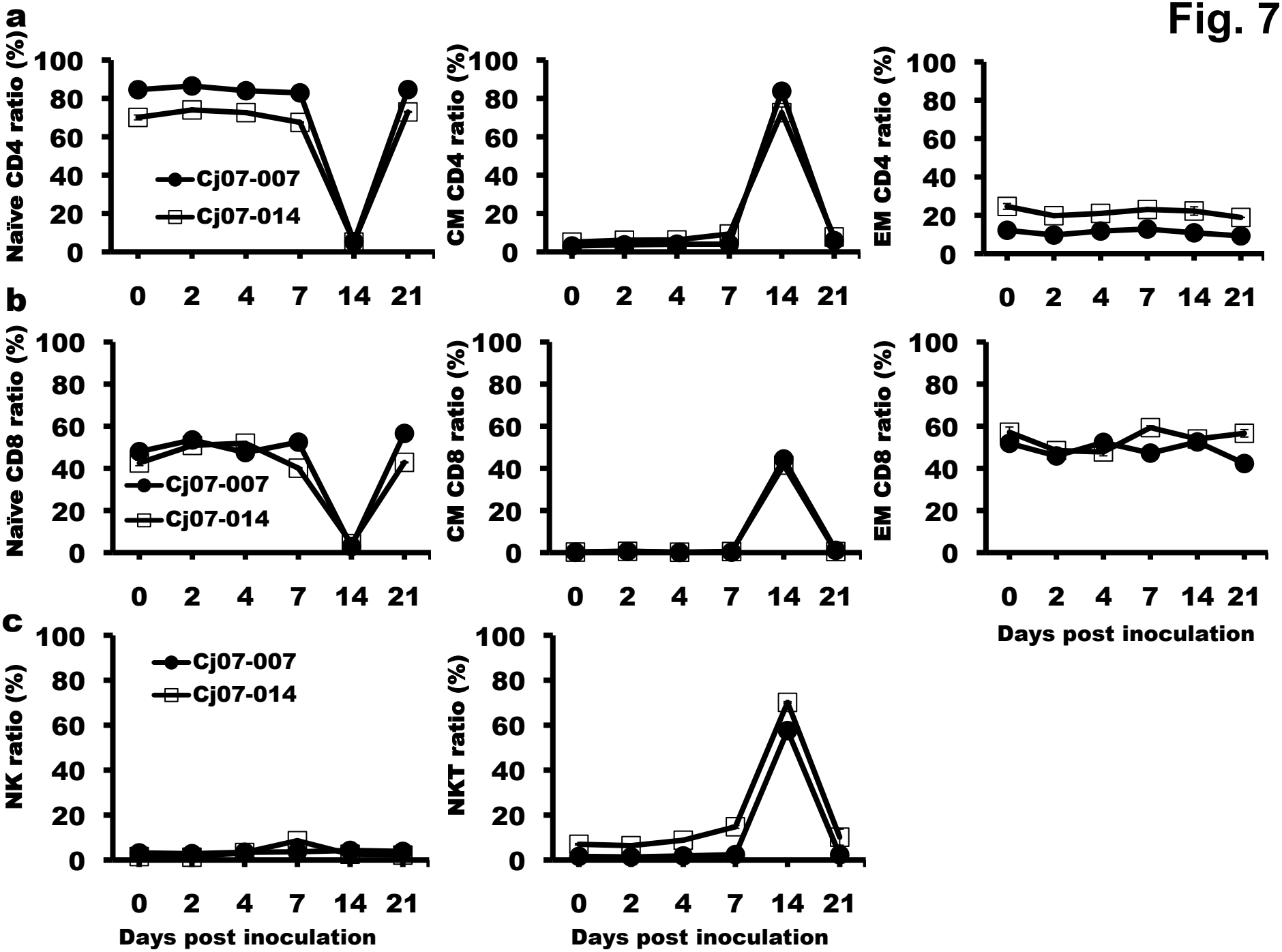
a
Naïve CD4 ratio (%)
CM CD4 ratio (%)
EM CD4 ratio (%)
b
Naïve CD8 ratio (%)
CM CD8 ratio (%)
EM CD8 ratio (%)
c
NK ratio (%)
NKT ratio (%)
Cj07-007
Cj07-014
0
20
40
60
80
100
0 2 4 7 14 21
Days post inoculation

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
$CD3^+CD4^+CD62L^-CD95^\pm$	(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
$CD3^+CD8^+CD62L^-CD95^\pm$	(CD8 T_{EM})	28.8±14.8
$CD3^-CD16^+$	(NK)	4.2±2.6
$CD3^+CD16^+$	(NKT)	5.1±3.4

SD: Standard deviation.
Results shown are mean±SD from 3 healthy marmosets.

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

Subpopulation name		Subpopulation Ratios (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
$CD3^+CD4^+CD62L^-CD95^±$	(CD4 T_{EM})	12.8±0.9	9.5±1.0	12.3±0.4	12.3±0.5	13.4±3.2	18.9±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±7.9	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±0.8	3.8±1.2
$CD3^+CD8^+CD62L^-CD95^±$	(CD8 T_{EM})	7.8±1.6	5.6±0.8	9.0±4.1	9.5±3.1	27.6±7.2	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±0.4	3.2±1.7
$CD3^+CD16^+$	(NKT)	0.2±0.0	52.6±17	46.1±8.5	1.1±0.5	1.7±0.5	1.2±0.2

SD: Standard deviation.

Results shown are mean±SD from 2 marmosets as shown in Figure 3.

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	Day 14	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
$CD3^+CD4^+CD62L^+CD95^+$	(CD4 T_{CM})	13.9±1.3	17.5±4.1	15.2±2.5	40.0±13	33.8±10	21.3±12
$CD3^+CD4^+CD62L^-CD95^\pm$	(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
$CD3^+CD8^+CD62L^+CD95^-$	(CD8 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
$CD3^+CD8^+CD62L^+CD95^+$	(CD8 T_{CM})	3.9±0.6	7.4±2.8	5.5±1.6	28±17	8.1±4.6	8.6±8.9
$CD3^+CD8^+CD62L^-CD95^\pm$	(CD8 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.

Supplementary Figure Legends

Supplementary Figure 1. Levels of DENV RNA in primary or re-challenge DENV-infected marmosets. Data for these graphs was extracted from the study of Omatsu T. *et al.* (2011). Marmosets were subcutaneously infected with the DENV-2 Mal/77/08 strain or with the DENV-2 DHF0663 strain. The vRNAs were detected in plasma by real-time PCR. (a) Cj08-007, Cj07-011: Mal/77/08 strain ($1.9x10^5$ PFU/ml). At the primary DENV infection, two marmosets were inoculated subcutaneously in the back with 1.9 $x10^5$ PFU of the DENV-2 Mal/77/08 strain. (b) Cj07-006, Cj07-008: DHF0663 strain ($1.8x10^4$ PFU/ml). At the primary DENV infection, two marmosets were inoculated subcutaneously in the back with $1.8x10^4$ PFU of the DENV-2 DHF0663 strain. (c) Cj07-007, Cj07-014: DHF0663 strain ($1.8x10^5$ PFU/ml). In the case of the DENV re-challenge study, two marmosets initially inoculated with $1.8x10^5$ PFU of the DHF0663 strain were re-inoculated 33 weeks after the primary challenge with $1.8x10^5$ PFU of the same virus.

Supplementary materials and methods

Animals

All animal studies were conducted in accordance with the 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 6 male marmosets, weighing 258-512 g, were used. DENV infection status in marmosets was used from a previous study (Supplementary Figure 1) [2]. Marmosets were caged individually at 27±2 °C in 50±10% humidity with a 12h light-dark cycle (lighting from 7:00 to 19:00) at Tsukuba

Primate Research Center, National Institute of Biomedical Innovation, Tsukuba, Japan. All animals were fed twice a day with a standard marmoset diet supplemented with fruit, eggs and milk. Water was given ad libitum. The animals were in a healthy condition and confirmed to be negative for anti-dengue virus antibodies before inoculation with dengue virus [2].

Cells

Cell culture was performed as previously described [2]. 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 strains were reported as previously described [2]. DENV type 2 (DENV-2), DHF0663 strain (Accession no. AB189122) strain was 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 Maldives. The DENV-2 isolated clinical samples were propagated with C6/36 cells and were used within 4 passages on C6/36 cells. Culture supernatant from infected C6/36 cells was centrifuged at 3,000 rpm for 5 min to remove cell debris, and then stored at -80 °C until use.

Infection of marmosets with DENV

In the challenge study, the profiling of the key adaptive and innate immune cells in the

marmosets after serotype 2 of DENV (DENV-2) infection was examined. At the primary DENV infection, four marmosets were inoculated subcutaneously in the back with 1.9 $x10^5$ plaque forming unit (PFU) of the DENV-2 Mal/77/08 strain (Cj08-007, Cj07-011) or with $1.8x10^4$ PFU of the DENV-2 DHF0663 strain (Cj07-006, Cj07-008) [2]. In the case of the DENV re-challenge study, two marmosets initially inoculated with $1.8x10^5$ PFU of the DHF0663 strain were re-inoculated 33 weeks after the primary challenge with $1.8x10^5$ PFU of the same virus (Cj07-007, Cj07-014) [2]. 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 was performed under anesthesia with 5 mg/kg of ketamine hydrochloride. Day 0 was defined as the day of virus inoculation. DENV viral loads in marmosets were used from a previous study (Supplementary Figure 1) [2].

Titration of viral RNA in plasma

Plasma samples were stored at -80 °C until use. Viral RNA was isolated from plasma samples, using the High Pure Viral RNA Kit (Roche Diagnostics). Levels of dengue viral RNA were determined by TaqMan real time reverse transcriptase-PCR (TaqMan RT-PCR) as previously reported [1]. One PFU/ml of the DENV-2 DHF0663 strain from plasma samples was equivalent to 285 ± 35.4 copies/ml with this method, and the detection limit was $5x10^3$ copies/ml in plasma samples.

Supplementary References

1. Ito M, Takasaki T, Yamada K, Nerome R, Tajima S, Kurane I (2004) Development and evaluation of fluorogenic TaqMan reverse transcriptase PCR assays for detection of dengue virus types 1 to 4. J Clin Microbiol 42:5935-5937

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Supplementary Fig. 1