-BRIEF REPORT-

CD16 positive natural killer cells play a limited role against primary dengue virus infection in tamarins

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Key words: Dengue virus, tamarin, NK cells, CD16

Abstract

CD16 is a major molecule expressed on NK cells. To directly assess the role of natural killer (NK) cells in dengue virus (DENV) infection *in vivo*, CD16 antibody-treated tamarins were inoculated with DENV-2 strain. This resulted in the transient depletion of $CD16^+$ NK cells, whereas no significant effects on the overall levels or kinetics of plasma viral loads and anti-viral antibodies were observed in the treated monkeys as compared to those in the control monkeys. It remains elusive whether $CD16^-$ NK subpopulation could play an important role in the control of primary DENV infection.

DENV is one of the most serious mosquito-borne virus affecting humans with 2.5 billion people at risk in tropical and subtropical regions around the world each year [12]. A wide variety of clinical manifestations have been noted, which range from asymptomatic, mild febrile illness (dengue fever [DF]) to dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS), a life-threatening illness. It has been shown that humans with a secondary heterologous DENV infection are at a higher risk of contracting severe dengue disease [10, 26]. DHF/DSS occurs in infants during primary DENV infection predominantly in the second half of the first year of life when maternal antibodies have low residual neutralizing activity [11, 17].

NK cells are a component of the innate immune system that plays a central role in host defense against viral infection and tumor cells. It has been shown that infection by some viruses, such as herpes simplex virus-1, influenza virus or the ectromelia poxvirus, can be controlled by NK cells in mice [15]. Yet the most compelling evidence for a role of NK cells in early defense against viruses was obtained in a study showing increased susceptibility or resistance to murine cytomegalovirus (MCMV) after NK cell depletion or NK cell adoptive transfer, respectively [23]. Defects in NK cell activity, such as decreased production of interferon (IFN)-γ or cytotoxicity, render mice more susceptible to MCMV infection [23]. NK cells can kill virus-infected cells by using cytotoxic granules or by recognizing and inducing lyses of antibody-coated target cells (antibody-dependent cell cytotoxicity) via Fc binding receptor such as CD16 [21].

Early activity of NK cells may be important for clearing primary DENV infection [24]. In a DENV mouse model, mice experimentally infected with DENV showed increased NK cell levels [24]. A significant increase in the frequency of NK cell circulation was also shown in patients who developed an acute dengue disease [2]. In addition, patients with a mild dengue disease have elevated NK cell rates when compared to those with severe dengue diseases [9, 27]. Moreover, Kurane *et al.* reported that human blood NK cells are cytotoxic against DENV-infected cells in target organs via direct cytolysis and antibody-dependent cell-mediated cytotoxicity [14]. It was also shown that the intracellular cytotoxic granule, TIA-1, was up-regulated early in NK cells in the acute phase of DENV infection and that NK-activating receptor NKp44 was involved in virus-mediated NK activation through direct interaction with DENV envelope protein [2, 13]. These results suggest that the early activation of NK cells contributes to the prevention of the severe dengue disease. However, based on quantitative and functional analyses in animal model *in vivo*, defining the contribution of NK cells to suppress DENV replication *in vivo* has been necessary.

We have recently reported that common marmosets (*Callithrex jacchus*) are highly permissive to DENV infection [22]. The New World monkeys, being nonhuman primates are considered to have a similar immune system to humans [28, 29]. The present study was initiated to investigate the role of NK cells in controlling DENV during primary infection in our nonhuman primate model.

The animals were cared for in accordance with National Institute of Biomedical Innovation rules and guidelines for experimental animal welfare, and all protocols were approved by our Institutional Animal Study Committee. Eight tamarins (*Saguinus midas* and *Saguinus labiatus*) were used in this study. As marmosets and tamarins are closely related monkey species and are classified into Callitrichinae, we expected that tamarins would also be permissive to DENV infection as well as marmosets. To check the permissiveness of tamarins to DENV, 2 tamarins were infected with DENV-2 (DHF0663 strain: 6.7×10^7 PFU/ml) subcutaneously or intravenously (Fig. 1). Dengue viral RNA (vRNA), which was quantified using real-time PCR as previously described [22], was detected in plasma samples from the tamarins on day 1 post-infection. For each of the two tamarins (Tm03-011, Tm06-017), the plasma vRNA levels reached 2.7×10^6 copies/ml and 2.0×10^7 copies/ml on day 1 post-infection, respectively, and were detectable on days 3 and 5. These results indicate that tamarins are also permissive to DENV infection, which is consistent with the results obtained by using marmosets [22].

Next, we sought to assess the role of NK cells in DENV infection *in vivo*. In this regard, in vivo depletion of NK cells by the administration of NK-specific monoclonal antibody (mAb) was considered to be straightforward to directly address the question. We employed a new method by which an anti-CD16 mAb 3G8 [7] but not a control mAb MOPC-21 efficiently depleted a major NK population expressing CD16 in tamarins, as we recently reported [29]. The mouse anti-human CD16 mAb 3G8 was produced in serum-free medium and purified using protein A affinity chromatography. Endotoxin levels were confirmed to be lower than 1 EU/mg. Four red-handed tamarins and two white-lipped tamarins (*Saguinus labiatus*) were used in this experiment. Three tamarins were intravenously administered 3G8 at a dose of 50 mg/kg, while others were given a control mAb MOPC-21. One day later, both mAb-treated tamarins were subcutaneously inoculated with 3×10^5 PFU/ml of DENV-2 DHF0663 strain on the basis of a previous report that a single mosquito might inject between 10^4 and 10^5 PFU of DENV into a human [20]. It was confirmed that at 1-3 days after the 3G8 mAb treatment, $CD16^+$ cells were almost completely depleted in the tamarins followed by recovery to the initial levels at around 2 weeks after administration, while the cells were

maintained at the initial levels in the monkeys with MOPC-21 (Fig. 2A). In addition, it is noteworthy that the ratios of $CD4^+$ and $CD8^+$ T cells and $CD20^+$ B cells were not affected by the administration of the 3G8 mAb (Supplementary Figure 1). In the case of the MOPC-21 mAb administration, we confirmed no significant effect on $CD16^+$ cells (Supplementary Figure 2). The killing activities of the peripheral blood mononuclear cells (PBMCs) taken from the 3G8-treated monkeys were reduced at day 1 post antibody-treatment, followed by increase irrespective of depletion of $CD16^+$ NK cells at day 2 post antibody-treatment (1 day after DENV inoculation), suggesting that $CD16^-$ NK population may be activated by DENV infection (Fig. 2B). Plasma viral loads in both mAb-treated monkeys rose to 10^5 copies/ml by day 1 after infection and then reached a peak at 10^6 copies/ml on day 3 or day 7 followed by a rapid decline with values dipping below the detectable level by day 14 after infection (Fig. 2C). These results suggested that $CD16^+$ NK cells did not apparently contribute to DENV replication in the acute phase in our tamarin model.

It was previously reported that non-structural glycoprotein NS1 was essential for flavivirus viability and the NS1 protein circulated during the acute phase of disease in the plasma of patients infected with DENV [1]. Epidemiological studies have demonstrated that secreted NS1 levels are correlated with viremia levels and are higher in DHF than in dengue fever (DF) early in illness [16], thus it has been suggested that NS1 might be a useful marker as an indicator of the severity of dengue disease. We have used the value of the NS1 antigens as an alternative diagnostic marker to examine the effects of CD16 antibody treatment on DENV replication. The NS1 was measured by Platelia Dengue NS1 Ag assay (BioRad). Antigenemia was noted in these infected monkeys between 3-14 days post-infection. Serum IgM and IgG specific for DENV antigens were measured by ELISA. DENV specific IgM or IgG antibody was equally detected in both mAb-treated monkeys.

We recently demonstrated that marmosets were permissive to DENV infection [22]. In this study, we found that tamarins were also permissive to DENV infection (Fig.1). Moreover, we also investigated the role of NK cells against early DENV infection using *in vivo* depletion of $CD16^+$ NK cells in tamarins. As a result, the depletion of $CD16^+$ NK cells had almost no effect on DENV replication (Fig. 2), and thus the NK subpopulation was unlikely to contribute to controlling DENV replication. Interestingly, these results imply that $CD16^-$ NK subpopulation may have a critical role of controlling DENV infection *in vivo*.

Using our model, we investigated the roles of NK cells *in vivo* against DENV

infection, which remains to be elucidated in several aspects. We previously reported that almost complete *in vivo* depletion of $CD16^+$ NK subpopulation was not able to completely remove the NK-mediated cytotoxic activity in tamarins [29]. In this study, despite a transient but substantial reduction in $CD16^+$ NK cell number following 3G8 treatment in tamarins, DENV replication was comparable to that in monkeys with the control mAb. The NK-mediated cytotoxic activity was rather augmented in either study group, which indicates that $CD16^-$ NK cells was responsible for the cytotoxic activity and suggests that they might play a role in controlling DENV replication.

The next question is how $CD16^-$ NK cells may regulate DENV infection. One possibility regarding $CD16^-$ NK cells is that $CD56^+$ or $CD57^+$ NK cells are involved in controlling DENV infection. Human NK cells are classically divided into 2 functional subsets based on their cell surface density of CD56 and CD16, i.e. $CD56^{bright}CD16^-$ immunoregulatory cells and $CD56^{dim}CD16^+$ cytotoxic cells. Both subsets have been characterized extensively regarding their different functions, phenotypes, and tissue localization [8]. The NK cell number is maintained by a continuous differentiation process associated with the expression of CD57 that ends in NK cells with poor responsiveness to cytokine stimulation but high cytolytic capacity [3, 18]. The second possibility is that $CD16^-$ NK cells have non-cytolytic helper function. Generally, it is well known that NK cells possess both a cytolytic and a non-cytolytic helper function. It was suggested that cytokine production is carried out by $CD56^{bright}CD16^-$ NK cells [4-6]. Interferon (IFN)-γ secreted by NK cells has shown potent antiviral effects against DENV infection in early phases [25]. One aspect of the NK helper function arises from recent evidence indicating that NK cells can be induced to function as non-cytotoxic helper cells following stimulation with interleukin-18 [19]. This cytokine induces IFN-γ secretion from NK cells and thus enables dendritic cells (DCs) to secrete IL-12, leading to Th1 polarization [19]. It is possible that $CD16^-$ NK cells, which have poor cytotoxic activity but the enhanced ability to secrete cytokines and then to lead Th1 response, are preserved during 3G8 administration. The persistence of this minor $CD16^-$ NK cell subpopulation could exert an antiviral effect through INF-γ-mediated pathways despite the depletion of $CD16^+$ NK cells. The third possibility is that $CD16^+$ NK cells of tamarins play pivotal roles against bacterial infections and cancer progression but not DENV-infected cells. We will address these possibilities for the roles of the NK subpopulation in the future studies.

In conclusion, this study provides DENV replication model *in vivo* in tamarins and new information on the possible role of $CD16^+$ NK cells in DENV

194 replication *in vivo*. It remains elusive whether $CD16^+$ and $CD16^-$ NK subpopulation
195 could play an important role in the control of primary DENV infection.
196

Acknowledgements

We would like to give special thanks to members of the Corporation for Production and Research of Laboratory Primates for technical assistance. We also would like to give special thanks to Ms. Tomoko Ikoma and Ms. Mizuho Fujita for technical assistance. Moreover, we appreciate Dr. Keith A. Reimann (the NIH Nonhuman Primate Reagent Resource R24 RR016001, NIAID contact HHSN272200900037C) for providing CD16 and CD8 antibodies. This work was supported by grants from the Ministry of Health, Labor and Welfare of Japan (to Hirofumi Akari and Ichiro Kurane). This research was also supported by the environment Research and Technology Development Fund (D-1007) from the Ministry of the Environment of Japan (to Tomoyuki Yoshida and Hirofumi Akari).

207 **Figure legends**

208 **Fig. 1** Levels of vRNA in DENV-infected tamarins. Tamarins were subcutaneously or
209 intravenously infected with DENV at a dose of $6.7x10^7$ PFU/ml. The vRNAs were
210 detected in plasma by real-time PCR. Tm03-011: Subcutaneously. Tm06-017:
211 Intravenously.
212
213 **Fig. 2** Ratios of $CD16^+$ NK cells, killing activity of PBMCs, and vRNA in
214 DENV-infected tamarins after treatment of 3G8 or MOPC-21 mAb.
215 Tamarins were subcutaneously infected with DENV at a dose $3x10^5$ PFU/ml after
216 treatment with 50mg/kg of 3G8 or MOPC-21 mAb. (A) Ratios of $CD16^+$ NK cells were
217 determined in whole blood specimens. (B) The activities of NK cells were determined
218 in PBMCs of tamarins by NK cytotoxic assay. (C) The vRNAs were detected in plasma
219 by real-time PCR.
220
221 **Fig. 3** Levels of NS1 antigen and DENV-specific IgM and IgG in plasma samples from
222 DENV-infected tamarins after treatment of 3G8 or MOPC-21 mAb.
223 The levels of NS1 antigen and DENV-Specific IgM and IgG in plasma were measured
224 by ELISA. ELISA index of NS1 antigen (A), the positive/negative (P/N) ratio of
225 DENV-specific IgM (B), and P/N ratio of DENV-specific IgG (C) in plasma samples
226 from DENV-infected tamarins after administration of the 3G8 or MOPC-21 mAb. The
227 P/N ratio was calculated by the formula: the optical density of the test sample divided
228 by that of a negative sample. P/N ratios of <2 and ≥ 2 were considered to be negative
229 and positive, respectively. Top: 3G8, Bottom: MOPC-21 mAb.
230

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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.

Fig. 1

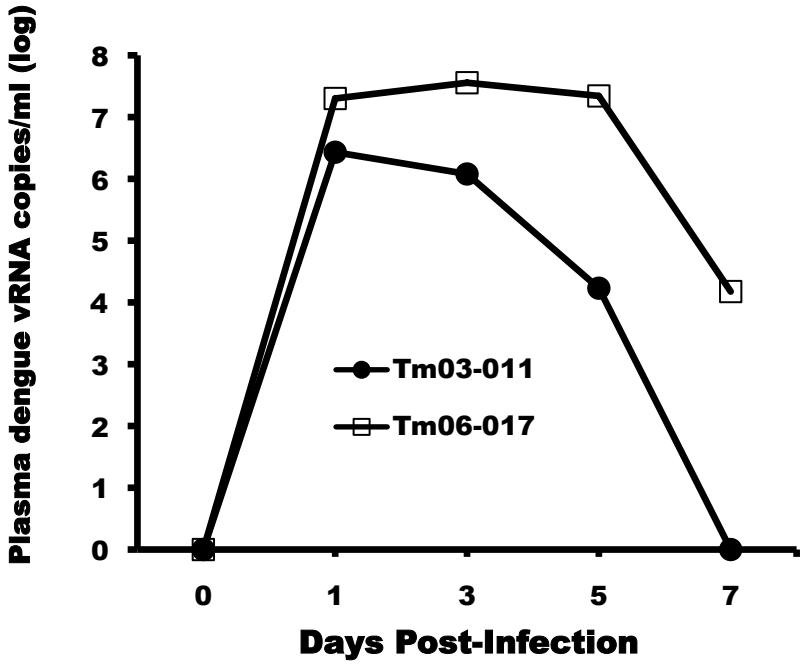

Plasma dengue vRNA copies/ml (log)
8
7
6
5
4
3
2
1
0
0
1
3
5
7
Days Post-Infection
Tm03-011
Tm06-017

Fig. 2

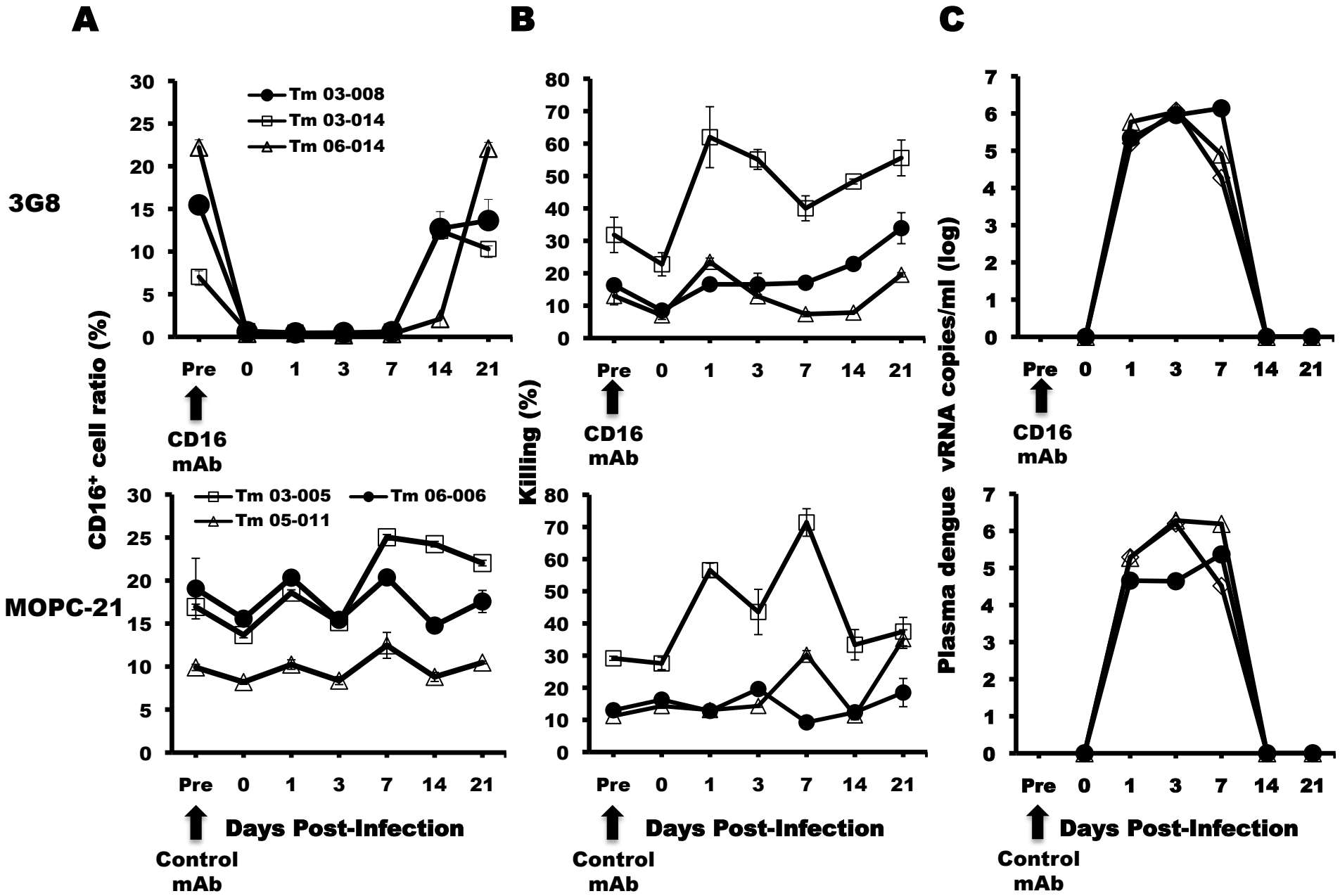
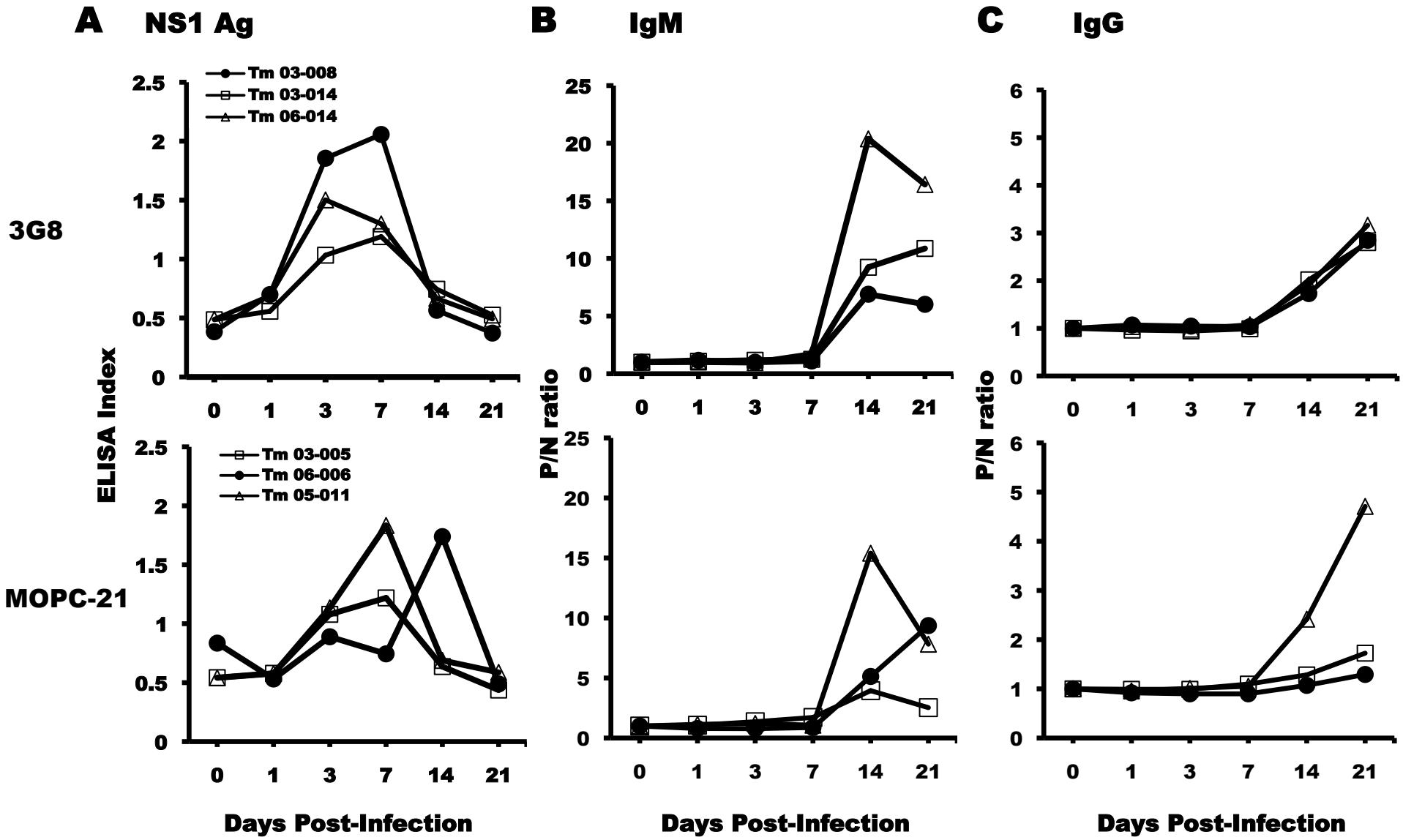

A
B
C
3G8
MOPC-21
CD16+ cell ratio (%)
Killing (%)
Plasma dengue vRNA copies/ml (log)
Tm 03-008
Tm 03-014
Tm 06-014
Tm 03-005
Tm 06-006
Tm 05-011
Pre
0
1
3
7
14
21
CD16 mAb
Control mAb
Days Post-Infection

Fig. 3


A
NS1 Ag
B
IgM
C
IgG
3G8
MOPC-21
Tm 03-008
Tm 03-014
Tm 06-014
Tm 03-005
Tm 06-006
Tm 05-011
ELISA Index
P/N ratio
P/N ratio
0
0.5
1
1.5
2
2.5
0
5
10
15
20
25
0
1
2
3
4
5
6
0 1 3 7 14 21
Days Post-Infection
Days Post-Infection
Days Post-Infection

Supplementary Legends

Supplementary Figure 1. Ratios of $CD4^+$ and $CD8^+$ T cells and $CD20^+$ B cells after administration of CD16 (3G8) antibody *in vivo* in tamarin.

(A-C) Tamarin was administered with 50 mg/kg of 3G8 mAb. $CD4^+$ and $CD8^+$ T cells and $CD20^+$ B cells were determined in whole blood specimens. Tamarin: Tm 03-008.

Supplementary Figure 2. Control antibody (MOPC-21) did not deplete $CD16^+$ cells *in vivo* in tamarins. (A, B) Tamarins were administered with 50 mg/kg of the control antibody (MOPC-21). $CD16^+$ NK cell numbers were determined in whole blood specimens. Tamarins: Tm 03-012, Tm 06-013.

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. One tamarin was used. Tamarins were 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. Animal was fed twice a day with a standard tamarin diet supplemented with fruit, eggs and milk. Water was given ad libitum. All animals were in a healthy condition and confirmed to be negative for anti-dengue virus antibodies before inoculation with dengue virus [3].

Cells

Cell culture was performed as previously described [3]. 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 strain was reported as previously described [3]. 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 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.

***In vivo* depletion of CD16 positive cells**

Mouse anti-human CD16 (3G8) mAb [2] and a mouse immunoglobulin G (IgG1κ) isotype-matched irrelevant control antibody (MOPC-21) were produced in serum-free medium and purified using protein A affinity chromatography [1]. Endotoxin levels were lower than 1EU/mg. Administration of CD16 antibody (3G8) or control antibody (MOPC-21) was performed as previously described [4]. The each antibody was administered to tamarin (Tm 03-008, Tm 03-012, Tm 06-013) intravenously at 50 mg/kg at a rate of 18 ml/min using a syringe pump. Lymphocyte subsets were monitored for 2~3 weeks after the administration.

Infection of marmosets with DENV

In the challenge study, profiling of the key adaptive and innate immune cells in marmosets after serotype 2 of DENV (DENV-2) was examined. Marmosets were inoculated subcutaneously in the back with $1.8x10^4$ or $1.8x10^5$ PFU of the DENV-2 DHF0663 strain [3]. Blood samples were collected on day 0, 1, 3, 7, 14, and 21 after inoculation. Blood samples were used for FACS 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.

Flow cytometry

Flow cytometry was performed as previously described [4]. Fifty microliters of whole blood from tamarins was stained with combinations of fluorescence-conjugated monoclonal antibodies (mAb): anti-CD3 (SP34-2; Becton Dickinson), anti-CD4 (L200; BD Pharmingen), anti-CD8 (CLB-T8/4H8; Sanquin), anti-CD16 (3G8; BD Pharmingen) and anti-CD20 (H299; BECKMAN COULTER). Then, erythrocytes were lysed with FACS lysing solution (Becton Dickinson). After having been washed with sample buffer containing phosphate-buffered saline (PBS), 1% fetal calf serum (FCS), and 1% formaldehyde, the labeled cells were resuspended in the sample buffer. The expression of the immunolabeled molecules on the lymphocytes was analyzed with a FACSCanto II flow cytometer (Becton Dickinson).

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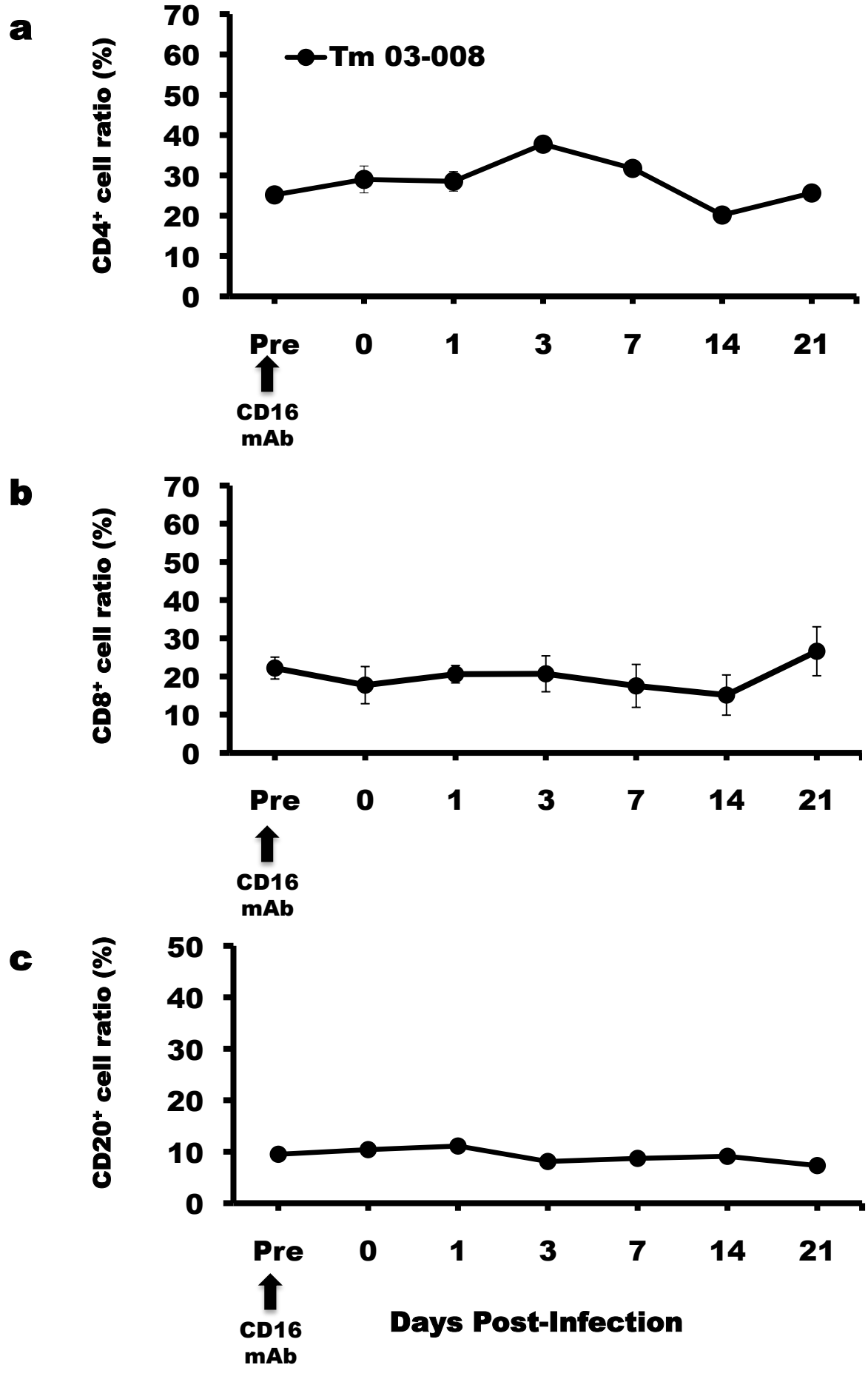
Supplementary Fig. 1



Supplementary Fig. 2