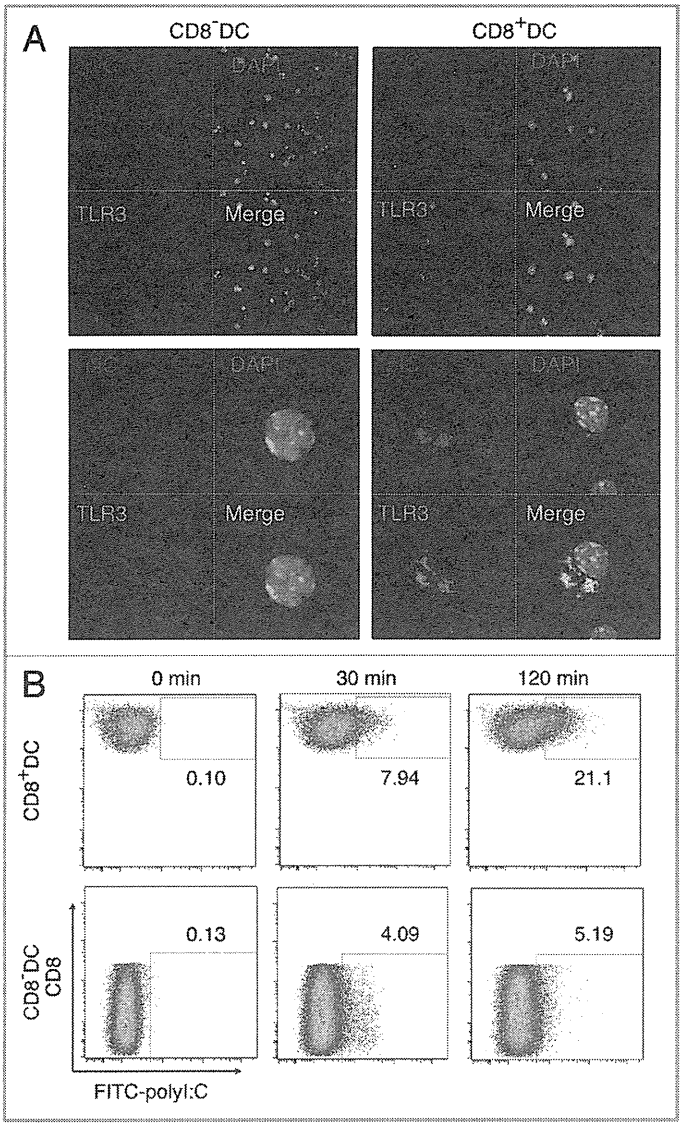

Figure 6. PolyI:C encounters TLR3 in CD8α⁺ DC. CD8α⁺ and CD8α⁻ DC were isolated by FACSAriaII and stimulated with 20 μg/ml TexasRed-polyI:C for 2 h. Then cells were stained with Alexa647-antiTLR3 and subjected to confocal microscopic analysis (A). Alternatively, splenic DC isolated by MACS were incubated with FITC-polyI:C for the time shown in figure and analyzed the degrees of polyI:C uptake by FACS (B). Data shown are the representative of three independent experiments.

as soluble Ags, the TICAM-1 pathway in CD8α⁺ DC would be crucial for driving of tumor-specific CTL around the tumor microenvironment. In any route of polyI:C injection, this is true as shown first in this study. Although TICAM-1 is an adaptor of other cytoplasmic sensors, DDX1, DDX21 and DHX36,[32] the antitumor CTL responses are merely relied on TLR3 of CD8α⁺DC in this system. Taken together with previous reports,[11,12] TICAM-1 signaling triggers not only NK activation but also CTL induction.

TLR3 and MDA5 are main sensors for dsRNA and differentially distributed in myeloid cells.[33,34] TLR3 is limitedly expressed in myeloid, epithelial and neuronal cells,[33] whereas MDA5 is ubiquitously expressed including non-myeloid stromal cells.[33] Several reports suggested that i.v. injection of polyI:C predominantly stimulate the stromal cells which express IFNAR,[26] thereby robust type I IFN are liberated from these cells to be a systemic response including cytokinemia and endotoxin-like shock.[35,36] Both TLR3 and MDA5 link to the IRF-3/7-activating kinases leading to the production of IFNα/β.[37,38] Once IFNα/β are released, IFNAR senses it to amplify the Type I IFN production,[39] and reportedly this amplification pathway involves cross-priming of CD8 T cells in viral infection.[18] Tumor progression or metastasis can be suppressed through the IFNAR pathway.[40] These scenarios may be right depending on the conditions employed. Our message is related to what signal pathway is fundamentally required for induction of antitumor CTL in DC. The CTL response is almost completely abrogated in TICAM-1$^{-/-}$ and IRF-3/7$^{-/-}$ mice, but largely remains in IPS-1$^{-/-}$ and IFNAR$^{-/-}$ mice when Ag and polyI:C are extrinsically administered. The results are reproducible in some other tumor-implant models (data not shown), and even in IFNAR$^{-/-}$ mice, TICAM-1-specific genes are upregulated to confer tumor cytotoxicity (**Fig. S6**, Azuma et al., unpublished data). In addition, the upregulation of these genes is independent of IPS-1 knockout in DC. Our results infer that the primary sensing of dsRNA in CD8α⁺ DC is competent to induce cross-presentation, which minimally involves the IPS-1 or IFNAR amplification pathway, at least at a low dose of polyI:C. Yet, subsequent induction of Type I IFN via the IFNAR may further amplify the cross-priming.[18,41] Further studies are needed as to which of the TICAM-1-inducible genes link to the cross-presentation in CD8α⁺ DC.

The main focus of this study was to identify the pathway for transversion of immature DC to the CTL-driving phenotype by co-administration of polyI:C with soluble Ag. The IPS-1 pathway, although barely participates in antitumor CTL driving, can upregulate CD40/CD86 co-stimulators on the membranes of splenic CD8α⁺ and CD8α⁻ DC in response to polyI:C, suggesting that MDA5 does function in the cytoplasm of splenic CD8α⁺ and CD8α⁻ DC to sense polyI:C. However, effective CTL induction happens only in CD8α⁺ DC when stimulated with polyI:C. CD8α⁺ DC express TLR3 but CD8α⁻ DC do not, and CD8α⁺ DC with no TLR3 fail to induce CTL, suggesting that integral co-stimulation by MDA5/IPS-1 is insufficient for DC to induce cross-priming of CD8 T cells: antitumor CTL are not induced until the TICAM-1 signal is provided in DC. At least, sole effect of the IPS-1 pathway and upregulation of co-stimulators on CD8α⁺ DC is limited for cross-priming and induction of antitumor CTL, which result partly reflects those in a previous report where IPS-1 and TICAM-1 harbor a similar potential for CD8 T cell proliferation when

polyI:C (Alum-containing) is employed as an adjuvant for CD8α+ DC to test proliferation of anti-OVA CTL.[21]

A question is why TICAM-1 is dominant to IPS-1 for response to exogenously-added polyI:C in CD8α+ DC. The answer is rooted in the difference of functional behavior between BMDC and CD8α+ DC. TLR3 levels are variable depending upon subsets of DC,[22] which affects DC subset-specific induction of cellular immune response. The high TLR3 expression (partly surface-expressed) is situated in CD8α+ DC before polyI:C stimulation, which is distinct from the properties of F4/80+ Mf and presumably BMDC of low TLR3 expression. The polyI:C-uptake machinery[15] appears to efficiently work in concert with the TLR3/TICAM-1 pathway in CD8α+ DC and this tendency is diminished when CD8α+ DC are pretreated with Alum + polyI:C.[21] Furthermore, there are functional discrepancies between CD8α+ splenic DC and GM-CSF-induced BMDC, which appears to reflect the difference of their TLR3 levels.[22] These results on CD8α+ DC encourage us to develop dsRNA adjuvant immunotherapy supporting TAA soluble vaccines for cancer applicable to humans, which possess the counterpart of CD8α+ DC.

There are two modes of dsRNA-mediated DC maturation, intrinsic and extrinsic modes that are governed by the IPS-1 and TICAM-1 pathways, respectively.[9,34] It is important to elucidate the in vivo qualitative difference in the two pathways in tumor-loading mice. TLR3+ DC/Mf are responsible for CTL driving via an extrinsic route in viral infection.[34] Previous data suggested that dsRNA in infectious cell debris, rather than viral dsRNA produced in the cytoplasm of Ag-presenting cells or autophagosome formation, contribute to fine tuning of DC maturation through extrinsic dsRNA recognition.[16] It is reported that dsRNA-containing debris are generated secondary to infection-mediated cell death,[41] and DC phagocytose by-stander dead cells. Likewise, soluble tumor Ags released from tumor cells usually are extrinsically taken up by APC in patients with cancer.[42] If CTL are successfully induced in therapeutic biotherapy targeted against cancer cells, this extrinsic TICAM-1 pathway must be involved in the therapeutic process.

Cross-presentation occurs in a TAP-dependent[43] and -independent fashions.[44,45] The peptides are transported by TAP into the endoplasmic reticulum (ER) and loaded onto MHC Class I for presentation at the cell surface. ER and phagosome might fuse each other for accelerating cross-presentation.[46] Another possibility is that cross-presentation occurs in early endosomes where TLR3 resides. This early endosome cross-presentation does not always depend on TAP[42-44] but requires TLR stimulation.[34] TLR4/MyD88 pathway is involved in the TAP-dependent early endosome model,[43] where recruitment of TAP to the early endosomes is an essential step for the cross-presentation of soluble Ag. These models together with our genechip analysis of polyI:C-stimulated BMDC suggested that some ER-associated proteins are upregulated in BMDC by polyI:C-TICAM-1 pathway. The results infer that the TLR3/TICAM-1 rather than the TLR4/MyD88 pathway more crucially participates in cross-presentation in response to dsRNA or viral stimuli and facilitates raising CTL antitumor immunity in APC.

Although multiple RNA sensors couple with TICAM-1 and signal to activate the Type I IFN-inducing pathway,[25] at least TLR3 in the CD8α+ DC are critical in CTL driving. CD8α+ DC are a high TLR3 expresser, while BMDC express TLR3 with only low levels.[22] CD8α- DC do not express it.[22] The Ag presentation and TLR3 levels in CD8α+ DC appear reciprocally correlated with the phagocytosing ability of DC. Although the TLR3 mRNA level is downregulated secondary to polyI:C response after maturation, this may not be related to the CD8α+ DC functions. Yet, polyI:C might interact with other cytoplasmic sensors for DC maturation,[32,47]

The route of administration and delivery methods may be important for culminate the polyI:C adjuvant function. The toxic problem has not overcome in the adjuvant therapy using polyI:C[35,36] and this is a critical matter for clinical introduction of dsRNA reagents to immunotherapy. The most problematic is the life-threatening shock induced by polyI:C. Recent advance of polyI:C study suggests that PEI-jet helps efficient uptake of polyI:C into peritoneal macrophages.[48] LC (poly-L-lysine and methylcellulose) has been used as a preservative to reduce the toxic effect of polyI:C.[49] Nanotechnological delivery of polyI:C results in efficient tumor regression.[50] There are many subsets of DC that can be defined by surface markers, and selecting an appropriate administration route can target a specific DC subset. The route for s.c. administration usually mature dermal/epidermal DC or Langerhans cells.[51,52] Some DC subsets with unique properties specialized to CTL induction would work in association with the route of polyI:C administration. Attempting to develop more harmless and efficient dsRNA derivatives will benefit for establishing human adjuvant immunotherapy for cancer.

Materials and Methods

Mice. TICAM-1$^{-/-}$ and IPS-1$^{-/-}$ mice were made in our laboratory and backcrossed more than eight times to adapt C57BL/6 background.[12] IRF-3/7$^{-/-}$ and IFNAR$^{-/-}$ mice were kindly provided by T. Taniguchi (University of Tokyo, Tokyo, Japan). TLR3$^{-/-}$ mice were kindly provided by S. Akira (Osaka University, Osaka, Japan). Rag2$^{-/-}$ and OT-1 mice were kindly provided from Drs N. Ishii (Tohoku University, Sendai, Japan). Rag2$^{-/-}$/OT-1 mice were bred in our laboratory. All mice were maintained under specific pathogen-free conditions in the animal facility of the Hokkaido University Graduate School of Medicine. Animal experiments were performed according to the guidelines set by the animal safety center, Hokkaido University, Japan.

Cells. EG7 and C1498 cells were purchased from ATCC and cultured in RPMI1640/10% FCS/55 μM 2-ME/1 mM sodium pyruvate and RPMI1640/10% FCS/25 ng/ml 2-ME, respectively. Mouse splenocytes, OT-1 T cell, CD8α+ DC and CD8α- DC were harvested from the spleen and cultured in RPMI1640/10% FCS/55 μM 2-ME/10 mM HEPES.[41] B16D8 cells were cultured in RPMI/10% FCS as described previously.[12]

Reagents and antibodies. Ovalbumin (OVA) and polyI:C (polyI:C) were purchased from SIGMA and Amersham Biosciences, respectively. $OVA_{257-264}$ peptide (SIINFEKL: SL8)

and OVA ($H2K^b$-SL8) Tetramer were from MBL. Following Abs were purchased: anti-CD3ε (145-2C11), anti-CD8β (53-6.7), anti-CD11c (N418), anti-CD16/32 (93), anti-CD69 (H1.2F3) and anti-IFNγ(XMG1.2) Abs from BioLegend, anti-B220 (RA3-6B2), anti-CD4 (L3T4), anti-CD40 (1C10), anti-CD86 (GL1), and anti-MHC I-SL8 (25-D1.16) Abs from eBiosciences, anti-TCR-Vβ5.1/5.2 Ab and ViaProbe from BD Biosciences. The Rat anti-mouse TLR3 mAb (11F8) was kindly provided by David M. Segal (National Institute of Health, Bethesda, MD). To rule out LPS contamination, we treated OVA or other reagents with 200 μg/ml of Polymixin B for 30 min at 37°C before use. Texas Red- or FITC-labeled poly(I:C) was prepared using the 5' EndTag™ Nucleic Acid Labeling System (Vector Laboratories) according to the manufacturers instructions.

Tumor challenge and poly I:C therapy. Mice were shaved at the back and s.c. injected with 200 μl of 2×10^6 syngenic EG7 cells in PBS. Tumor volumes were measured at regular intervals by using a caliper. Tumor volume was calculated by using the formula: Tumor volume (cm^3) = (long diameter) × (short diameter)^2 × 0.4. A volume of 50 μl of a mixture consisting of the lysate of 2×10^5 EG7 cells with or without 50 μg of poly I:C (polyI:C) was s.c. injected around the tumor. We added no other emulsified reagent for immunization since we want to role out the conditional effect of the Ag/polyI:C. The treatments were started when the average of tumor volumes reached at 0.4–0.8 cm^3 and performed twice per week. EG7 lysate were prepared by three times freeze/thaw cycles (-140°C/37°C) in PBS, with removal of cell debris by centrifugation at 6,000 g for 10 min.[53] To deplete CD8 T cells, mice were i.p. injected with hybridoma ascites of anti-CD8β mAb. The dose of antibody and the treatment regimens were determined in preliminary studies by using the same lots of antibody used for the experiments. Depletion of the desired cell populations by this treatment was confirmed by FACS for the entire duration of the study.

Evaluation of T cell activity in tumor-bearing mice. Draining inguinal LN cells were harvested from tumor-bearing mice after 24 h from the last polyI:C treatment. The activity of T cells was evaluated by CD69 expression and IL-2/IFNγ production. These cells were stained with FITC-CD8α, PE-CD69, PerCP/Cy5.5-7AAD and APC-CD3ε. To check cytokine production, LN cells were cultured for 3 d in vitro in the presence or absence of EG7 lysates and IL-2 and IFNγ productions were determined by Cytokine Beads Array (CBA) assay (BD). To assess the cytotoxic activity of CTL, standard ^{51}Cr release assay was performed. For CTL expansion, 2.5×10^6 LN cells were co-cultured with 1.25×10^5 mitomycin C-treated EG7 cells in the presence of 10 U/ml IL-2 for 5 d. Then, LN cells were incubated with ^{51}Cr-labeled EG7 or C1498 cells for 4 h and determined cytotoxic activity. The cell-specific cytotoxicity was calculated with subtracting the cytotoxity for C1498 from for EG7 cells.

Antigen-specific T cell expansion in vivo. Mice were i.p. immunized with 1 mg of OVA and 150 μg of poly I:C. After 7 d, spleens were homogenized and stained with FITC-CD8α and PE-OVA Tetramer for detecting OVA-specific CD8 T cell populations. For intracellular cytokine detection, splenocytes were cultured with or without 100 nM OVA peptide (SIINFEKL; SL8) for 8 h and 10 μg/ml of Brefeldin A (Sigma-Aldrich) was added to the culture in the last 4 h. Then cells were stained with PE-anti-CD8α and fixed/permeabilized with Cytofix/Cytoperm (BD Biosciences) according to manufacturer's instruction. Then, fixed/permeabilized cells were further stained with APC-anti-IFNγ. Stained cells were analyzed with FACSCalibur (BD Biosciences) and FlowJo software (Tree Star).

In vivo CTL assay. The in vivo CTL assay was performed as described.[54] In brief, WT, TICAM-$1^{-/-}$, $MAVS^{-/-}$ and IRF-$3/7^{-/-}$ mice were i.v. administered with PBS, 10 μg of OVA or OVA with 50 μg of polyI:C. After 5 d, 2×10^7 target cells (see below) were i.v. injected to other irrelevant mice and 8 h later, the OVA-specific cytotoxicity was measured by FACSCalibur. Target cells were 1:1 mixture of 2 μM SL8-pulsed, 5 μM CFSE-labeled splenocytes and SL8-unpulesed, 0.5 μM CFSE-labeled splenocytes. OVA-specific cytotoxicity was calculated with a formula: {1-(Primed [$CFSE^{high}$(%)/$CFSE^{low}$(%)]/Unprimed [$CFSE^{high}$(%)/$CFSE^{low}$(%)]} × 100.

DC preparation. DCs were prepared from spleens of mice, as described previously.[55] In brief, collagenase-digested spleen cells were treated with ACK buffer and then washed with PBS twice. Then splenocytes were positively isolated with anti-CD11c MicroBeads. $CD11c^+$ cells were acquired routinely about ≥ 80% purity. Further, to highly purify $CD8\alpha^+$ and $CD8\alpha^-$ DCs, spleen DC were stained with FITC-CD8α, PE-B220, PE/Cy7-CD11c and PerCP5.5-7AAD. $CD8\alpha^+$ or $CD8\alpha^-$ $CD11c^+B220^-$ DCs were purified on FACSAriaII (BD). The purity of the cells was ≥ 98%.

OT-1 proliferation assay. Ten micrograms of OVA with or without 50 μg of polyI:C were i.v. injected to WT, TICAM-$1^{-/-}$, IPS-$1^{-/-}$ and DKO mice. After 4 h, $CD8\alpha^+$ or $CD8\alpha^-$ DC were purified from the spleen. 2.5×10^4 $CD8\alpha^+$ or $CD8\alpha^-$ DC were co-cultured with 5×10^4 1 μM CFSE-labeled $Rag2^{-/-}$/OT-1 T cells for 3 d in 96-well round bottom plate. These cells were stained with PE-anti-TCR-Vβ5.1,5.2 and APC-anti-CD3ε and T cell proliferation was analyzed by CFSE dilution using FACSCalibur. Additionally, IFNγ in the culture supernatant was measured by CBA assay.

Statistical analysis. P-values were calculated with one-way analysis of variance (ANOVA) with Bonferroni's test. Error bars represent the SD or SEM between samples.

Disclosure of Potenial Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgment

We are grateful to Drs. T. Taniguchi (University Tokyo, Tokyo), N. Ishii (Tohoku University, Sendai) and D.M. Segel (NCI, Bethesda) for providing us with IRF-$3/7^{-/-}$ mice, OT-1 mice and anti-monse TLR3 mAb, respectively. Invaluable discussions about the peptide vaccine therapy with Dr. N. Satoh (Sapporo Medical

University, Sapporo) are gratefully acknowledged. We thank Drs H. Takaki, J. Kasamatsu, H.H. Aly, and H. Shime in our lab for their critical comments on this study.

This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, and Culture (Specified Project for Advanced Research, MEXT) and the Ministry of Health, Labor, and Welfare of Japan, and by the Takeda and the Waxmann Foundations. Financial supports by a MEXT Grant-in-Project "The Carcinogenic Spiral" is gratefully acknowledged.

Supplemental Materials

Supplemental materials may be found here:
http://www.landesbioscience.com/journals/oncoimmunology/article/19893/

References

1. Iwasaki A, Medzhitov R. Regulation of adaptive immunity by the innate immune system. Science 2010; 327:291-5; PMID:20075244; http://dx.doi.org/10.1126/science.1183021
2. Seya T, Shime H, Ebihara T, Oshiumi H, Matsumoto M. Pattern recognition receptors of innate immunity and their application to tumor immunotherapy. Cancer Sci 2010; 101:313-20; PMID:20059475; http://dx.doi.org/10.1111/j.1349-7006.2009.01442.x
3. Akira S. Toll-like receptor signaling. J Biol Chem 2003; 278:38105-8; PMID:12893815; http://dx.doi.org/10.1074/jbc.R300028200
4. Kawai T, Akira S. The roles of TLRs, RLRs and NLRs in pathogen recognition. Int Immunol 2009; 21:317-37; PMID:19246554; http://dx.doi.org/10.1093/intimm/dxp017
5. Longman RS, Braun D, Pellegrini S, Rice CM, Darnell RB, Albert ML. Dendritic-cell maturation alters intracellular signaling networks, enabling differential effects of IFN-alpha/beta on antigen cross-presentation. Blood 2007; 109:1113-22; PMID:17018853; http://dx.doi.org/10.1182/blood-2006-05-023465
6. Shinohara ML, Kim JH, Garcia VA, Cantor H. Engagement of the type I interferon receptor on dendritic cells inhibits T helper 17 cell development: role of intracellular osteopontin. Immunity 2008; 29:68-78; PMID:18619869; http://dx.doi.org/10.1016/j.immuni.2008.05.008
7. Diebold SS. Recognition of viral single-stranded RNA by Toll-like receptors. Adv Drug Deliv Rev 2008; 60:813-23; PMID:18241955; http://dx.doi.org/10.1016/j.addr.2007.11.004
8. Matsumoto M, Oshiumi H, Seya T. Antiviral responses induced by the TLR3 pathway. Rev Med Virol 2011. Epub ahead of print. PMID:21312311; http://dx.doi.org/10.1002/rmv.680
9. Yoneyama M, Fujita T. RIG-I family RNA helicases: cytoplasmic sensor for antiviral innate immunity. Cytokine Growth Factor Rev 2007; 18:545-51; PMID:17683970; http://dx.doi.org/10.1016/j.cytogfr.2007.06.023
10. Seya T, Matsumoto M. The extrinsic RNA-sensing pathway for adjuvant immunotherapy of cancer. Cancer Immunol Immunother 2009; 58:1175-84; PMID:19184005; http://dx.doi.org/10.1007/s00262-008-0652-9
11. Akazawa T, Ebihara T, Okuno M, Okuda Y, Shingai M, Tsujimura K, et al. Antitumor NK activation induced by the Toll-like receptor 3-TICAM-1 (TRIF) pathway in myeloid dendritic cells. Proc Natl Acad Sci U S A 2007; 104:252-7; PMID:17190817; http://dx.doi.org/10.1073/pnas.0605978104
12. Ebihara T, Azuma M, Oshiumi H, Kasamatsu J, Iwabuchi K, Matsumoto K, et al. Identification of a polyI:C-inducible membrane protein that participates in dendritic cell-mediated natural killer cell activation. J Exp Med 2010; 207:2675-87; PMID:21059856; http://dx.doi.org/10.1084/jem.20091573
13. Perrot I, Deauvieau F, Massacrier C, Hughes N, Garrone P, Durand I, et al. TLR3 and Rig-like receptor on myeloid dendritic cells and Rig-like receptor on human NK cells are both mandatory for production of IFN-gamma in response to double-stranded RNA. J Immunol 2010; 185:2080-8; PMID:20639488; http://dx.doi.org/10.4049/jimmunol.1000532
14. Bevan MJ. Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. J Exp Med 1976; 143:1283-8; PMID:1083422; http://dx.doi.org/10.1084/jem.143.5.1283
15. Datta SK, Redecke V, Prilliman KR, Takabayashi K, Corr M, Tallant T, et al. A subset of Toll-like receptor ligands induces cross-presentation by bone marrow-derived dendritic cells. J Immunol 2003; 170:4102-10; PMID:12682240
16. Schulz O, Diebold SS, Chen M, Näslund TI, Nolte MA, Alexopoulou L, et al. Toll-like receptor 3 promotes cross-priming to virus-infected cells. Nature 2005; 433:887-92; PMID:15711573; http://dx.doi.org/10.1038/nature03326
17. Kono H, Rock KL. How dying cells alert the immune system to danger. Nat Rev Immunol 2008; 8:279-89; PMID:18340345; http://dx.doi.org/10.1038/nri2215
18. Le Bon A, Etchart N, Rossmann C, Ashton M, Hou S, Gewert D, et al. Cross-priming of CD8+ T cells stimulated by virus-induced type I interferon. Nat Immunol 2003; 4:1009-15; PMID:14502286; http://dx.doi.org/10.1038/ni978
19. Bennett SR, Carbone FR, Karamalis F, Miller JF, Heath WR. Induction of a CD8+ cytotoxic T lymphocyte response by cross-priming requires cognate CD4+ T cell help. J Exp Med 1997; 186:65-70; PMID:9206998; http://dx.doi.org/10.1084/jem.186.1.65
20. Shimizu K, Kurosawa Y, Taniguchi M, Steinman RM, Fujii S. Cross-presentation of glycolipid from tumor cells loaded with alpha-galactosylceramide leads to potent and long-lived T cell mediated immunity via dendritic cells. J Exp Med 2007; 204:2641-53; PMID:17923500; http://dx.doi.org/10.1084/jem.20070458
21. Kumar H, Koyama S, Ishii KJ, Kawai T, Akira S. Cutting edge: cooperation of IPS-1- and TRIF-dependent pathways in poly IC-enhanced antibody production and cytotoxic T cell responses. J Immunol 2008; 180:683-7; PMID:18178804
22. Jelinek I, Leonard JN, Price GE, Brown KN, Meyer-Manlapat A, Goldsmith PK, et al. TLR3-specific double-stranded RNA oligonucleotide adjuvants induce dendritic cell cross-presentation, CTL responses, and antiviral protection. J Immunol 2011; 186:2422-9; PMID:21242525; http://dx.doi.org/10.4049/jimmunol.1002845
23. Wang Y, Cella M, Gilfillan S, Colonna M. Cutting edge: polyinosinic:polycytidylic acid boosts the generation of memory CD8 T cells through melanoma differentiation-associated protein 5 expressed in stromal cells. J Immunol 2010; 184:2751-5; PMID:20164430; http://dx.doi.org/10.4049/jimmunol.0903201
24. Carbone FR, Bevan MJ. Induction of ovalbumin-specific cytotoxic T cells by in vivo peptide immunization. J Exp Med 1989; 169:603-12; PMID:2784478; http://dx.doi.org/10.1084/jem.169.3.603
25. Asano J, Tada H, Onai N, Sato T, Horie Y, Fujimoto Y, et al. Nucleotide oligomerization binding domain-like receptor signaling enhances dendritic cell-mediated cross-priming in vivo. J Immunol 2010; 184:736-45; PMID:20008287; http://dx.doi.org/10.4049/jimmunol.0900726
26. McCartney S, Vermi W, Gilfillan S, Cella M, Murphy TL, Schreiber RD, et al. Distinct and complementary functions of MDA5 and TLR3 in poly(I:C)-mediated activation of mouse NK cells. J Exp Med 2009; 206:2967-76; PMID:19995959; http://dx.doi.org/10.1084/jem.20091181
27. Watanabe A, Tatematsu M, Saeki K, Shibata S, Shime H, Yoshimura A, et al. Raftlin is involved in the nucleocapture complex to induce poly(I:C)-mediated TLR3 activation. J Biol Chem 2011; 286:10702-11; PMID:21266579; http://dx.doi.org/10.1074/jbc.M110.185793
28. Itoh K, Watanabe A, Funami K, Seya T, Matsumoto M. The clathrin-mediated endocytic pathway participates in dsRNA-induced IFN-beta production. J Immunol 2008; 181:5522-9; PMID:18832709
29. Talmadge JE, Adams J, Phillips H, Collins M, Lenz B, Schneider M, et al. Immunomodulatory effects in mice of polyinosinic-polycytidylic acid complexed with poly-L-lysine and carboxymethylcellulose. Cancer Res 1985; 45:1058-65; PMID:3155990
30. Conforti R, Ma Y, Morel Y, Paturel C, Terme M, Viaud S, et al. Opposing effects of toll-like receptor (TLR3) signaling in tumors can be therapeutically uncoupled to optimize the anticancer efficacy of TLR3 ligands. Cancer Res 2010; 70:490-500; PMID:20068181; http://dx.doi.org/10.1158/0008-5472.CAN-09-1890
31. Kaiser WJ, Offermann MK. Apoptosis induced by the toll-like receptor adaptor TRIF is dependent on its receptor interacting protein homotypic interaction motif. J Immunol 2005; 174:4942-52; PMID:15814722
32. Zhang Z, Kim T, Bao M, Facchinetti V, Jung SY, Ghaffari AA, et al. DDX1, DDX21, and DHX36 helicases form a complex with the adaptor molecule TRIF to sense dsRNA in dendritic cells. Immunity 2011; 34:866-78; PMID:21703541; http://dx.doi.org/10.1016/j.immuni.2011.03.027
33. Gitlin L, Barchet W, Gilfillan S, Cella M, Beutler B, Flavell RA, et al. Essential role of mda-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus. Proc Natl Acad Sci U S A 2006; 103:8459-64; PMID:16714379; http://dx.doi.org/10.1073/pnas.0603082103
34. Matsumoto M, Seya T. TLR3: interferon induction by double-stranded RNA including poly(I:C). Adv Drug Deliv Rev 2008; 60:805-12; PMID:18262679; http://dx.doi.org/10.1016/j.addr.2007.11.005

35. Absher M, Stinebring WR. Toxic properties of a synthetic double-stranded RNA. Endotoxin-like properties of poly I, poly C, an interferon stimulator. Nature 1969; 223:715-7; PMID:5805520; http://dx.doi.org/10.1038/223715a0
36. Berry LJ, Smythe DS, Colwell LS, Schoengold RJ, Actor P. Comparison of the effects of a synthetic polyribonucleotide with the effects of endotoxin on selected host responses. Infect Immun 1971; 3:444-8; PMID:16557994
37. Sasai M, Shingai M, Funami K, Yoneyama M, Fujita T, Matsumoto M, et al. NAK-associated protein 1 participates in both the TLR3 and the cytoplasmic pathways in type I IFN induction. J Immunol 2006; 177:8676-83; PMID:17142768
38. Ishikawa H, Barber GN. STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. Nature 2008; 455:674-8; PMID:18724357; http://dx.doi.org/10.1038/nature07317
39. Taniguchi T, Takaoka A. A weak signal for strong responses: interferon-alpha/beta revisited. Nat Rev Mol Cell Biol 2001; 2:378-86; PMID:11331912; http://dx.doi.org/10.1038/35073080
40. Ogasawara S, Yano H, Momosaki S, Akiba J, Nishida N, Kojiro S, et al. Growth inhibitory effects of IFN-beta on human liver cancer cells in vitro and in vivo. J Interferon Cytokine Res 2007; 27:507-16; PMID: 17572015; http://dx.doi.org/10.1089/jir.2007.0183
41. Ebihara T, Shingai M, Matsumoto M, Wakita T, Seya T. Hepatitis C virus-infected hepatocytes extrinsically modulate dendritic cell maturation to activate T cells and natural killer cells. Hepatology 2008; 48:48-58; PMID:18537195; http://dx.doi.org/10.1002/hep.22337
42. Chaput N, Conforti R, Viaud S, Spatz A, Zitvogel L. The Janus face of dendritic cells in cancer. Oncogene 2008; 27:5920-31; PMID:18836473; http://dx.doi.org/10.1038/onc.2008.270
43. Burgdorf S, Schölz C, Kautz A, Tampé R, Kurts C. Spatial and mechanistic separation of cross-presentation and endogenous antigen presentation. Nat Immunol 2008; 9:558-66; PMID:18376402; http://dx.doi.org/10.1038/ni.1601
44. Shen L, Sigal LJ, Boes M, Rock KL. Important role of cathepsin S in generating peptides for TAP-independent MHC class I crosspresentation in vivo. Immunity 2004; 21:155-65; PMID:15308097; http://dx.doi.org/10.1016/j.immuni.2004.07.004
45. Kurotaki T, Tamura Y, Ueda G, Oura J, Kutomi G, Hirohashi Y, et al. Efficient cross-presentation by heat shock protein 90-peptide complex-loaded dendritic cells via an endosomal pathway. J Immunol 2007; 179:1803-13; PMID:17641047
46. Gagnon E, Duclos S, Rondeau C, Chevet E, Cameron PH, Steele-Mortimer O, et al. Endoplasmic reticulum-mediated phagocytosis is a mechanism of entry into macrophages. Cell 2002; 110:119-31; PMID:12151002; http://dx.doi.org/10.1016/S0092-8674(02)00797-3
47. Samuel CE. Antiviral actions of interferons. Clin Microbiol Rev 2001; 14:778-809; PMID:11585785; http://dx.doi.org/10.1128/CMR.14.4.778-809.2001
48. Wu CY, Yang HY, Monie A, Ma B, Tsai HH, Wu TC, et al. Intraperitoneal administration of poly(I:C) with polyethylenimine leads to significant antitumor immunity against murine ovarian tumors. Cancer Immunol Immunother 2011; 60:1085-96; PMID:21526359; http://dx.doi.org/10.1007/s00262-011-1013-7
49. Longhi MP, Trumpfheller C, Idoyaga J, Caskey M, Matos I, Kluger C, et al. Dendritic cells require a systemic type I interferon response to mature and induce CD4+ Th1 immunity with poly IC as adjuvant. J Exp Med 2009; 206:1589-602; PMID:19564349; http://dx.doi.org/10.1084/jem.20090247
50. Kitano S, Kageyama S, Nagata Y, Miyahara Y, Hiasa A, Naota H, et al. HER2-specific T-cell immune responses in patients vaccinated with truncated HER2 protein complexed with nanogels of cholesteryl pullulan. Clin Cancer Res 2006; 12:7397-405; PMID:17189412; http://dx.doi.org/10.1158/1078-0432.CCR-06-1546
51. Kushwah R, Hu J. Complexity of dendritic cell subsets and their function in the host immune system. Immunology 2011; 133:409-19; PMID:21627652; http://dx.doi.org/10.1111/j.1365-2567.2011.03457.x
52. Asano K, Nabeyama A, Miyake Y, Qiu CH, Kurita A, Tomura M, et al. CD169-positive macrophages dominate antitumor immunity by crosspresenting dead cell-associated antigens. Immunity 2011; 34: 85-95; PMID:21194983; http://dx.doi.org/10.1016/j.immuni.2010.12.011
53. Galea-Lauri J, Wells JW, Darling D, Harrison P, Farzaneh F. Strategies for antigen choice and priming of dendritic cells influence the polarization and efficacy of antitumor T-cell responses in dendritic cell-based cancer vaccination. Cancer Immunol Immunother 2004; 53:963-77; PMID:15146294; http://dx.doi.org/10.1007/s00262-004-0542-8
54. Durand V, Wong SY, Tough DF, Le Bon A. Shaping of adaptive immune responses to soluble proteins by TLR agonists: a role for IFN-α/β. Immunol Cell Biol 2004; 82:596-602; PMID:15550117; http://dx.doi.org/10.1111/j.0818-9641.2004.01285.x
55. Yamazaki S, Okada K, Maruyama A, Matsumoto M, Yagita H, Seya T. TLR2-dependent induction of IL-10 and Foxp3+ CD25+ CD4+ regulatory T cells prevents effective anti-tumor immunity induced by Pam2 lipopeptides in vivo. PLoS One 2011; 6:e18833; PMID:21533081; http://dx.doi.org/10.1371/journal.pone.0018833

Arch Virol
DOI 10.1007/s00705-013-1618-6

ORIGINAL ARTICLE

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

Tomoyuki Yoshida · Tsutomu Omatsu · Akatsuki Saito · Yuko Katakai · Yuki Iwasaki · Terue Kurosawa · Masataka Hamano · Atsunori Higashino · Shinichiro Nakamura · Tomohiko Takasaki · Yasuhiro Yasutomi · Ichiro Kurane · Hirofumi Akari

Received: 13 June 2012 / Accepted: 12 December 2012

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

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

Electronic supplementary material The online version of this article (doi:10.1007/s00705-013-1618-6) contains supplementary material, which is available to authorized users.

T. Yoshida · Y. Iwasaki · T. Kurosawa · M. Hamano · Y. Yasutomi · H. Akari
Tsukuba Primate Research Center, National Institute of Biomedical Innovation, 1-1 Hachimandai, Tsukuba, Ibaraki 305-0843, Japan

T. Yoshida (✉) · A. Saito · A. Higashino · H. Akari (✉)
Center for Human Evolution Modeling Research, Primate Research Institute, Kyoto University, Inuyama, Aichi 484-8506, Japan
e-mail: yoshida.tomoyuki.4w@kyoto-u.ac.jp

H. Akari
e-mail: akari.hirofumi.5z@kyoto-u.ac.jp

Introduction

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

T. Omatsu · T. Takasaki · I. Kurane
Department of Virology I, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

Y. Katakai
Corporation for Production and Research of Laboratory Primates, 1-1 Hachimandai, Tsukuba, Ibaraki 305-0843, Japan

S. Nakamura
Research Center for Animal Life Science, Shiga University of Medical Science, Seta Tsukinowa-cho, Otsu, Shiga 520-2192, Japan

Published online: 05 February 2013

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

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

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

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

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

Materials and methods

Animals

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

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

Cells

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

Virus

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

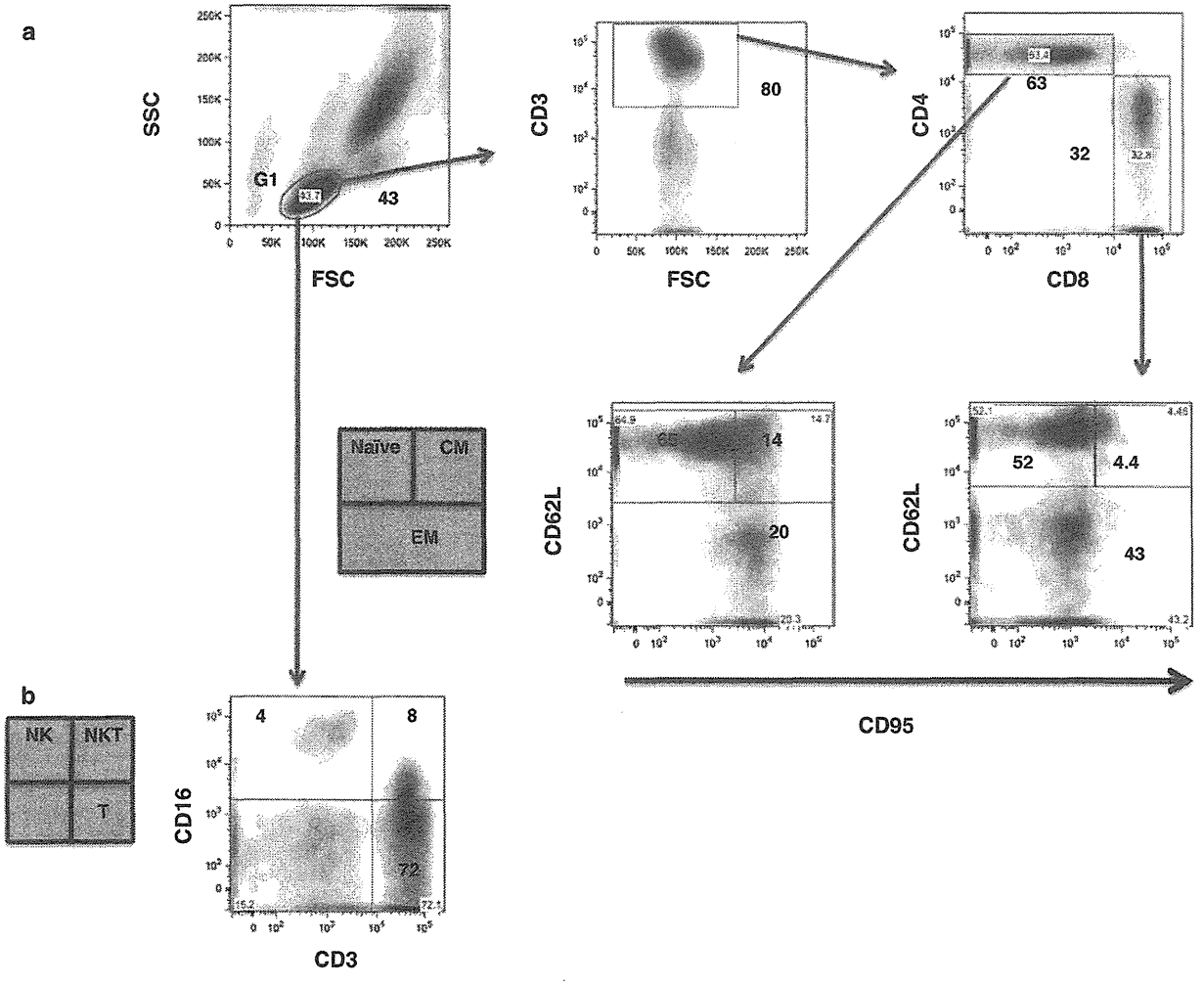

Fig. 1 Flow cytometric analysis of naïve, central/effector memory T cells and NK/NKT cells in marmosets. (**a**) Gating strategy to indentify CD4 and CD8 T, NK and NKT cells. The G1 population was selected and analyzed for CD4 and CD8 T, NK and NKT cells. (**a**) Profiling of naïve, central memory, and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

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

Infection of the marmosets with DENV

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

Flow cytometry

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

Table 1 Subpopulation ratios of lymphocytes in marmosets

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

SD: Standard deviation

Results shown are mean ± SD from 3 healthy marmosets

Results

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

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

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

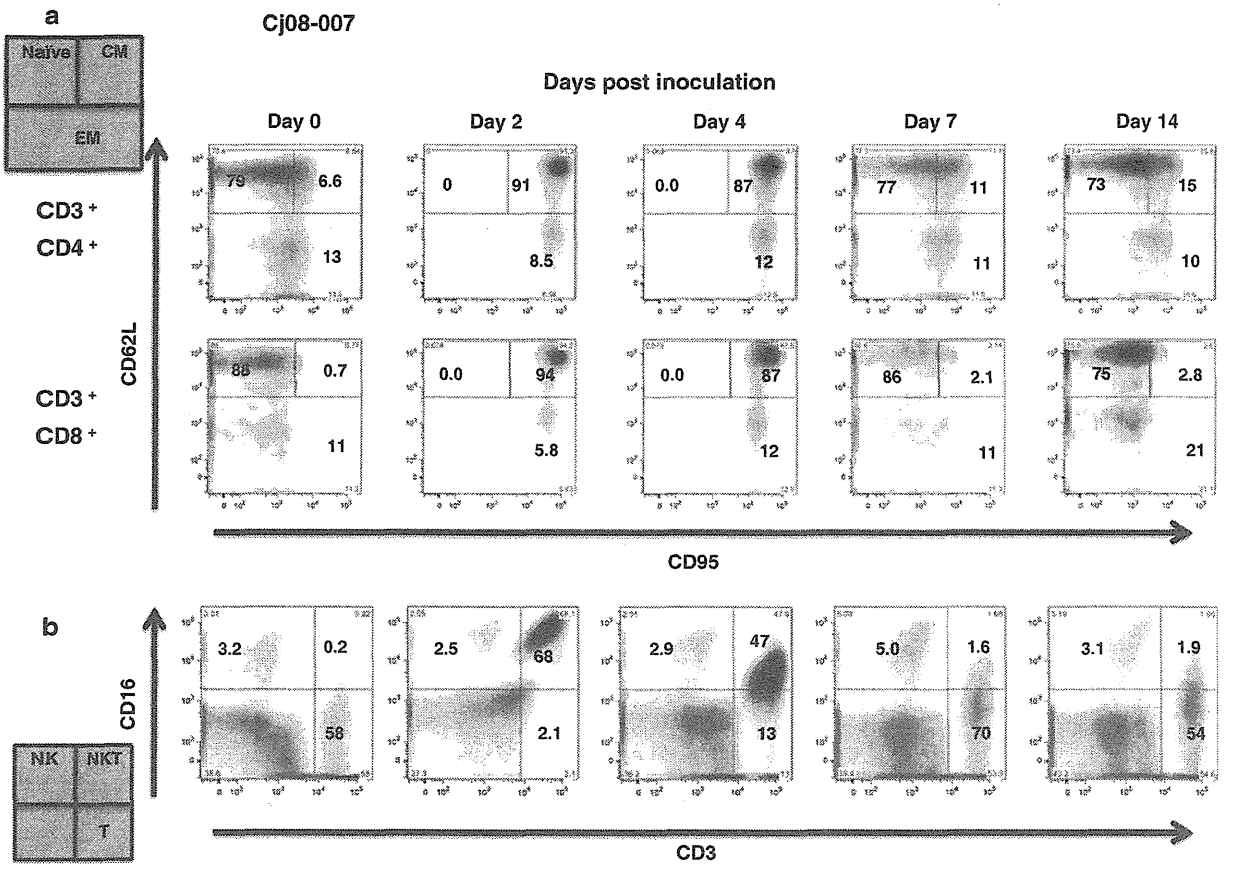

Fig. 2 Profiling of CD4 and CD8 T, NK and NKT cells in marmosets with primary infection with the DENV-2 Mal/77/08 strain. For primary DENV infection, two marmosets were inoculated subcutaneously in the back with 1.9×10^5 PFU of the DENV-2 Mal/77/08 strain. (**a**) Profiling of naïve, central memory,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

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

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

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

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

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

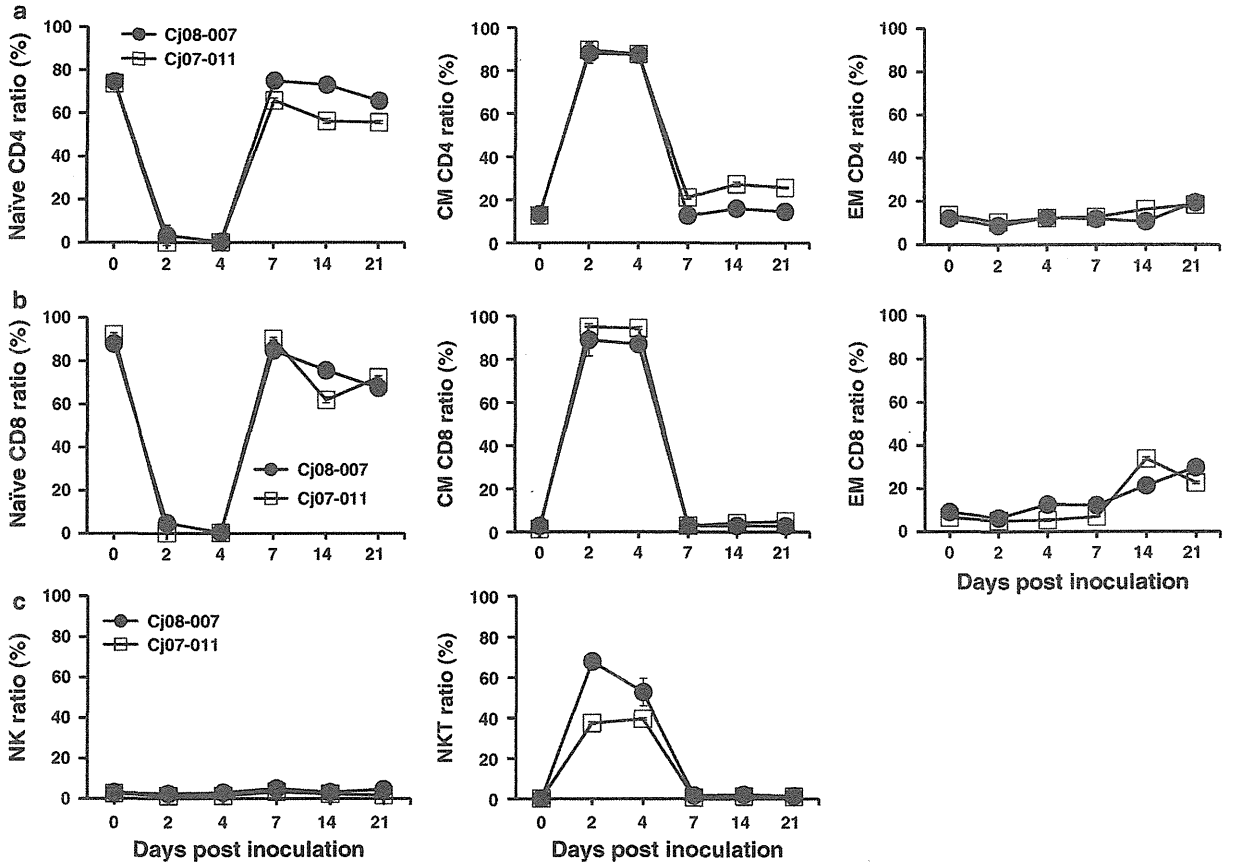

Fig. 3 Frequency of CD4 and CD8 T, NK and NKT cells in marmosets with primary infection with the DENV-2 Mal/77/08 strain. For primary DENV infection, two marmosets were inoculated subcutaneously in the back with 1.9×10^5 PFU of the DENV-2 Mal/77/08 strain. (**a**) Ratios of naïve, central memory, and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

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

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

SD: Standard deviation

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

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

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

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

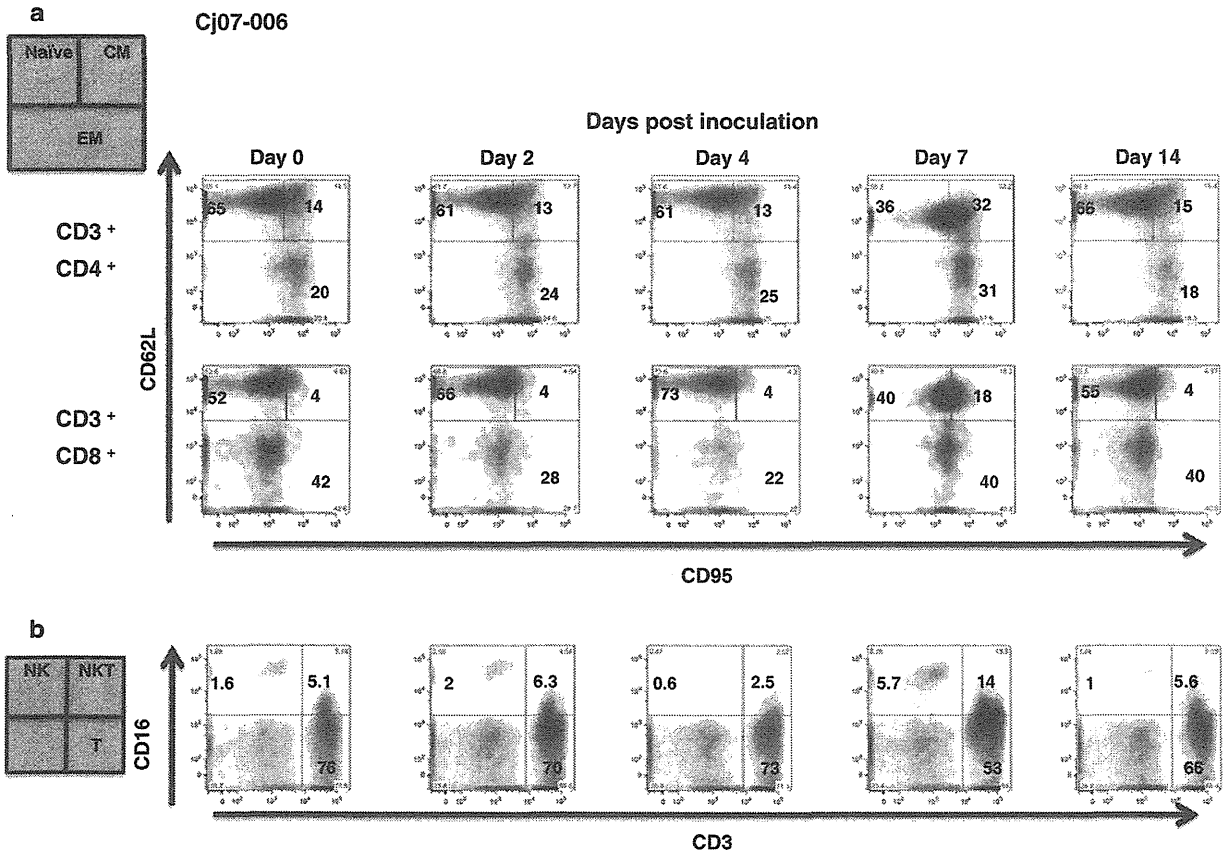

Fig. 4 Profiling of CD4 and CD8 T, NK and NKT cells in marmosets with primary infection with the DENV-2 DHF0663 strain. For primary DENV infection, two marmosets were inoculated subcutaneously in the back with 1.8×10^4 PFU of the DENV-2 DHF0663 strain. (**a**) Profiling of naïve, central memory, and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

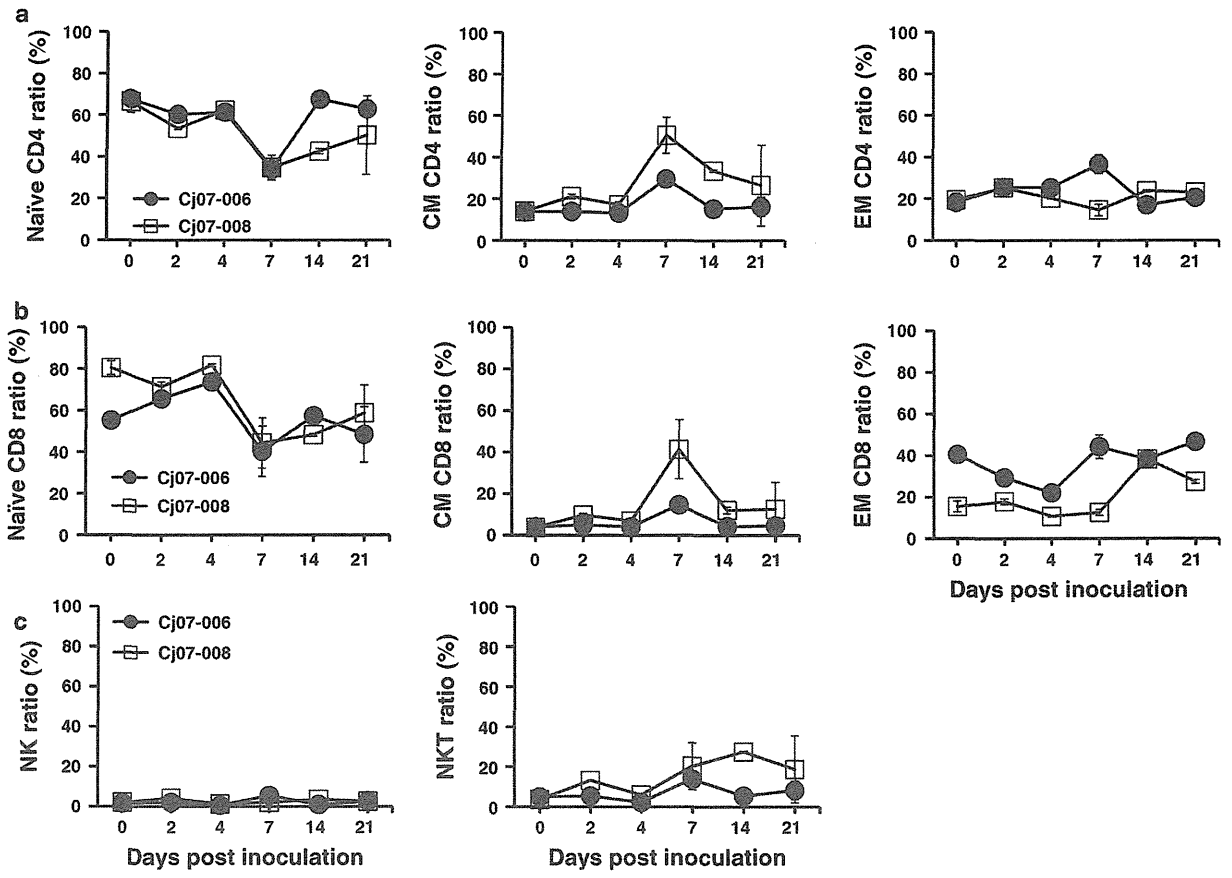

Fig. 5 Frequency of CD4 and CD8 T, NK and NKT cells in marmosets with primary infection with the DENV-2 DHF0663 strain. For primary DENV infection, two marmosets were inoculated subcutaneously in the back with 1.8×10^4 PFU of the DENV-2 DHF0663 strain. (**a**) Ratios of naïve, central memory, and effector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

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

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

SD: Standard deviation

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

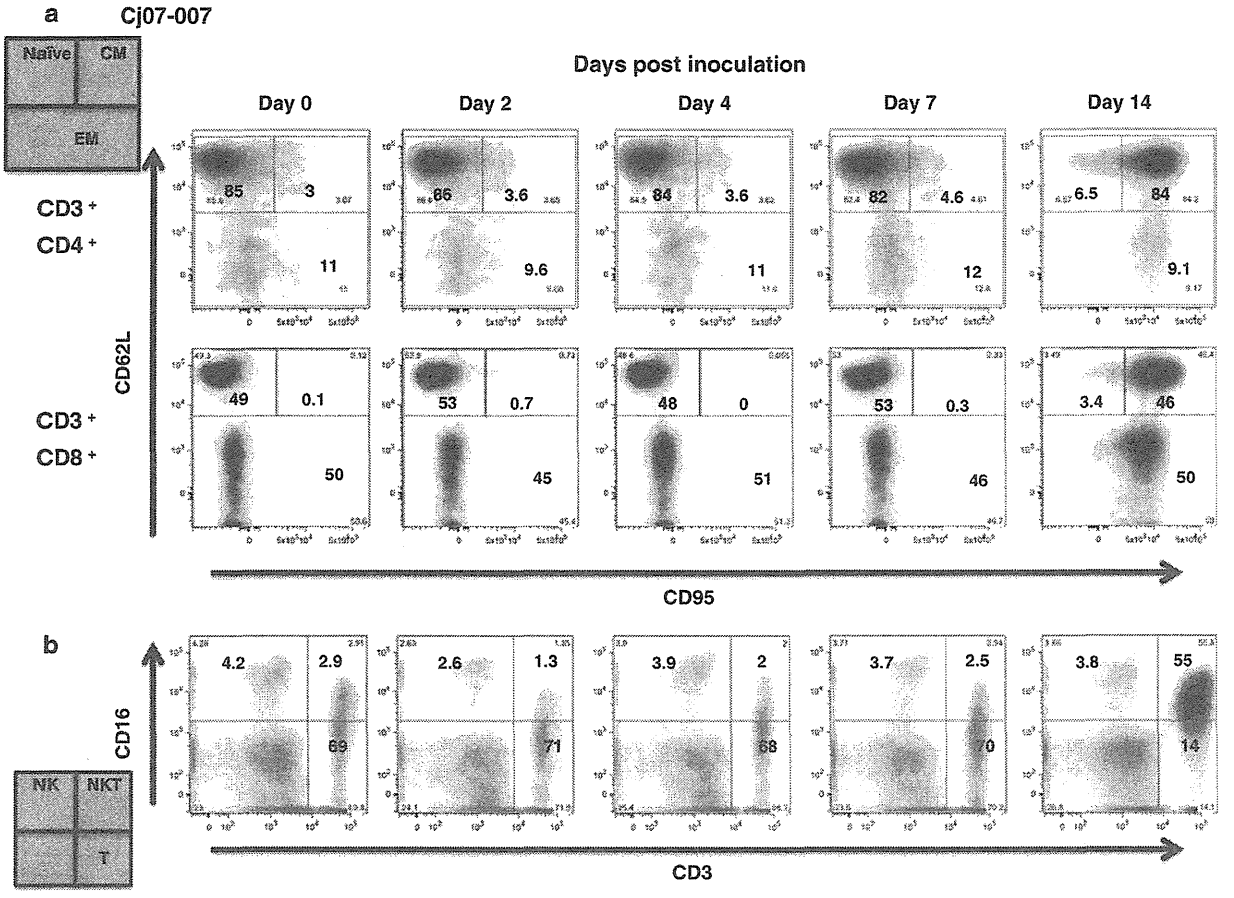

Fig. 6 Profiling of CD4 and CD8 T, NK and NKT cells in marmosets after re-challenge with the DENV-2 DHF0663 strain. Two marmosets that were initially inoculated with 1.8×10^5 PFU of the DHF0663 strain were re-inoculated 33 weeks after the primary challenge with 1.8×10^5 PFU of the same strain. (**a**)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

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

Discussion

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

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

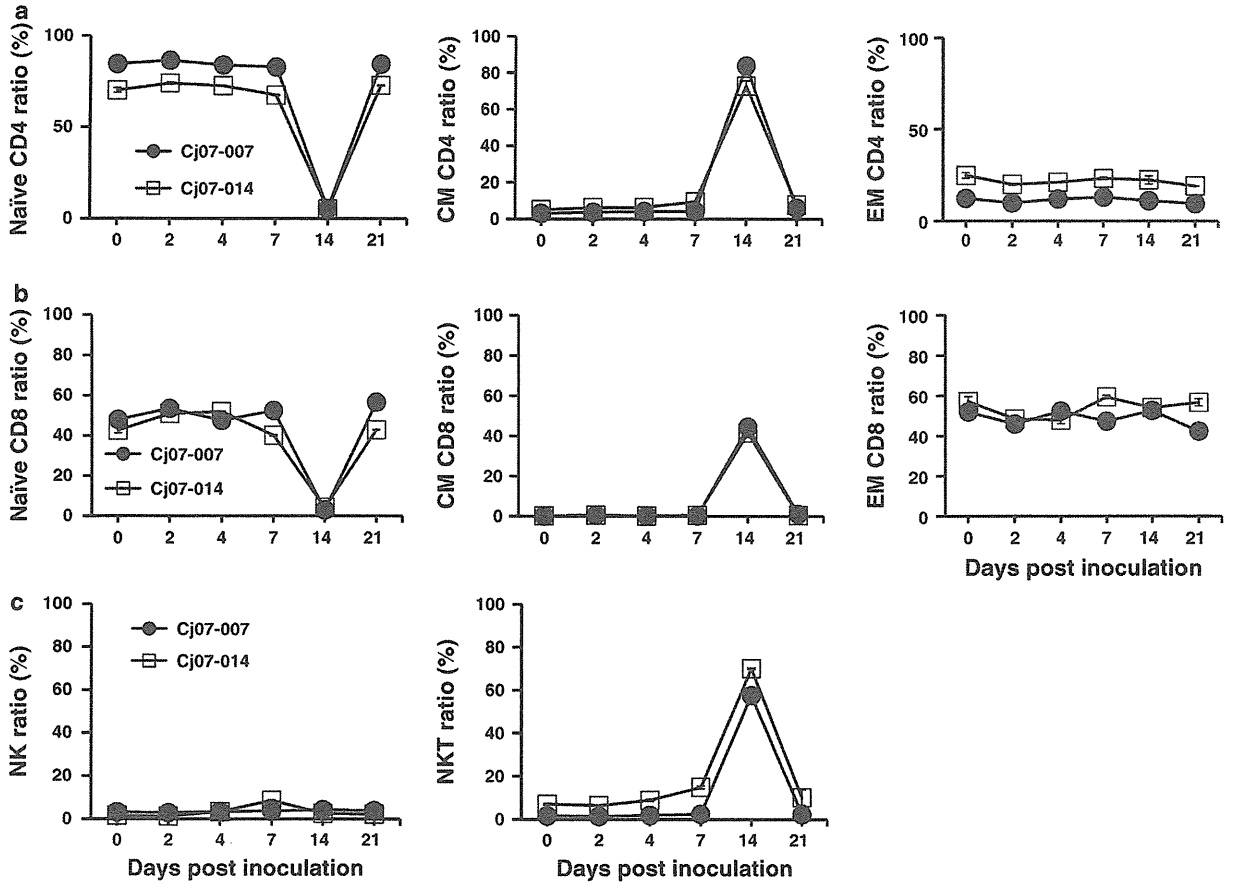

Fig. 7 Frequency of CD4 and CD8 T, NK and NKT cells in marmosets after re-challenge with the DENV-2 DHF0663 strain. Two marmosets initially inoculated with 1.8×10^5 PFU of the DHF0663 strain were re-inoculated 33 weeks after the primary challenge with 1.8×10^5 PFU of the same strain. (**a**) Ratios of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

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

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

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

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

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

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

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

References

1. Balsitis SJ, Williams KL, Lachica R, Flores D, Kyle JL, Mehlhop E, Johnson S, Diamond MS, Beatty PR, Harris E (2010) Lethal antibody enhancement of dengue disease in mice is prevented by Fc modification. PLoS Pathog 6:e1000790
2. Beaumier CM, Mathew A, Bashyam HS, Rothman AL (2008) Cross-reactive memory CD8(+) T cells alter the immune response to heterologous secondary dengue virus infections in mice in a sequence-specific manner. J Infect Dis 197:608–617
3. Beaumier CM, Rothman AL (2009) Cross-reactive memory CD4+ T cells alter the CD8+ T-cell response to heterologous secondary dengue virus infections in mice in a sequence-specific manner. Viral Immunol 22:215–219
4. Beaumier CM, Jaiswal S, West KY, Friberg H, Mathew A, Rothman AL (2010) Differential in vivo clearance and response to secondary heterologous infections by H2(b)-restricted dengue virus-specific CD8+ T cells. Viral Immunol 23:477–485
5. Fadilah SA, Sahrir S, Raymond AA, Cheong SK, Aziz JA, Sivagengei K (1999) Quantitation of T lymphocyte subsets helps to distinguish dengue hemorrhagic fever from classic dengue fever during the acute febrile stage. Southeast Asian J Trop Med Public Health 30:710–717
6. Goncalvez AP, Engle RE, St Claire M, Purcell RH, Lai CJ (2007) Monoclonal antibody-mediated enhancement of dengue virus infection in vitro and in vivo and strategies for prevention. Proc Natl Acad Sci USA 104:9422–9427
7. Green S, Pichyangkul S, Vaughn DW, Kalayanarooj S, Nimmannitya S, Nisalak A, Kurane I, Rothman AL, Ennis FA (1999) Early CD69 expression on peripheral blood lymphocytes from children with dengue hemorrhagic fever. J Infect Dis 180: 1429–1435
8. Green S, Vaughn DW, Kalayanarooj S, Nimmannitya S, Suntayakorn S, Nisalak A, Lew R, Innis BL, Kurane I, Rothman AL, Ennis FA (1999) Early immune activation in acute dengue illness is related to development of plasma leakage and disease severity. J Infect Dis 179:755–762
9. Gupta S, Gollapudi S (2008) CD95-mediated apoptosis in naive, central and effector memory subsets of CD4+ and CD8+ T cells in aged humans. Exp Gerontol 43:266–274
10. Guzman MG, Alvarez M, Rodriguez-Roche R, Bernardo L, Montes T, Vazquez S, Morier L, Alvarez A, Gould EA, Kouri G, Halstead SB (2007) Neutralizing antibodies after infection with dengue 1 virus. Emerg Infect Dis 13:282–286
11. Halstead SB (1979) In vivo enhancement of dengue virus infection in rhesus monkeys by passively transferred antibody. J Infect Dis 140:527–533
12. Halstead SB (2007) Dengue. Lancet 370:1644–1652
13. Henchal EA, Henchal LS, Schlesinger JJ (1988) Synergistic interactions of anti-NS1 monoclonal antibodies protect passively immunized mice from lethal challenge with dengue 2 virus. J Gen Virol 69(Pt 8):2101–2107
14. Hus I, Staroslawska E, Bojarska-Junak A, Dobrzynska-Rutkowska A, Surdacka A, Wdowiak P, Wasiak M, Kusz M, Twardosz A, Dmoszynska A, Rolinski J (2011) CD3+/CD16+CD56+ cell numbers in peripheral blood are correlated with higher tumor burden in patients with diffuse large B-cell lymphoma. Folia Histochem Cytobiol 49:183–187
15. Kaufman BM, Summers PL, Dubois DR, Eckels KH (1987) Monoclonal antibodies against dengue 2 virus E-glycoprotein protect mice against lethal dengue infection. Am J Trop Med Hyg 36:427–434
16. Kaufman BM, Summers PL, Dubois DR, Cohen WH, Gentry MK, Timchak RL, Burke DS, Eckels KH (1989) Monoclonal

antibodies for dengue virus prM glycoprotein protect mice against lethal dengue infection. Am J Trop Med Hyg 41:576–580
17. Khvedelidze M, Chkhartishvili N, Abashidze L, Dzigua L, Tsertsvadze T (2008) Expansion of CD3/CD16/CD56 positive NKT cells in HIV/AIDS: the pilot study. Georgian Med News 165:78–83
18. Kyle JL, Balsitis SJ, Zhang L, Beatty PR, Harris E (2008) Antibodies play a greater role than immune cells in heterologous protection against secondary dengue virus infection in a mouse model. Virology 380:296–303
19. Mathew A, Rothman AL (2008) Understanding the contribution of cellular immunity to dengue disease pathogenesis. Immunol Rev 225:300–313
20. Mladinich KM, Piaskowski SM, Rudersdorf R, Eernisse CM, Weisgrau KL, Martins MA, Furlott JR, Partidos CD, Brewoo JN, Osorio JE, Wilson NA, Rakasz EG, Watkins DI (2012) Dengue virus-specific CD4+ and CD8+ T lymphocytes target NS1, NS3 and NS5 in infected Indian rhesus macaques. Immunogenetics 64:111–121
21. Mueller YM, Makar V, Bojczuk PM, Witek J, Katsikis PD (2003) IL-15 enhances the function and inhibits CD95/Fas-induced apoptosis of human CD4+ and CD8+ effector-memory T cells. Int Immunol 15:49–58
22. Murphy BR, Whitehead SS (2011) Immune response to dengue virus and prospects for a vaccine. Annu Rev Immunol 29:587–619
23. Myint KS, Endy TP, Mongkolsirichaikul D, Manomuth C, Kalayanarooj S, Vaughn DW, Nisalak A, Green S, Rothman AL, Ennis FA, Libraty DH (2006) Cellular immune activation in children with acute dengue virus infections is modulated by apoptosis. J Infect Dis 194:600–607
24. Omatsu T, Moi ML, Hirayama T, Takasaki T, Nakamura S, Tajima S, Ito M, Yoshida T, Saito A, Katakai Y, Akari H, Kurane I (2011) Common marmoset (Callithrix jacchus) as a primate model of dengue virus infection: development of high levels of viremia and demonstration of protective immunity. J Gen Virol 92:2272–2280
25. Omatsu T, Moi ML, Takasaki T, Nakamura S, Katakai Y, Tajima S, Ito M, Yoshida T, Saito A, Akari H, Kurane I (2013) Changes in hematological and serum biochemical parameters in common marmosets (Cllithrix jacchus) after inoculation with dengue virus. J Med Primatol 54:89–98
26. Onlamoon N, Noisakran S, Hsiao HM, Duncan A, Villinger F, Ansari AA, Perng GC (2010) Dengue virus-induced hemorrhage in a nonhuman primate model. Blood 115:1823–1834
27. Pawitan JA (2011) Dengue virus infection: predictors for severe dengue. Acta Med Indones 43:129–135
28. Pitcher CJ, Hagen SI, Walker JM, Lum R, Mitchell BL, Maino VC, Axthelm MK, Picker LJ (2002) Development and homeostasis of T cell memory in rhesus macaque. J Immunol 168:29–43
29. Rigau-Perez JG, Clark GG, Gubler DJ, Reiter P, Sanders EJ, Vorndam AV (1998) Dengue and dengue haemorrhagic fever. Lancet 352:971–977
30. Sabin AB (1950) The dengue group of viruses and its family relationships. Bacteriol Rev 14:225–232
31. Sierra B, Garcia G, Perez AB, Morier L, Rodriguez R, Alvarez M, Guzman MG (2002) Long-term memory cellular immune response to dengue virus after a natural primary infection. Int J Infect Dis 6:125–128
32. Terabe M, Berzofsky JA (2008) The role of NKT cells in tumor immunity. Adv Cancer Res 101:277–348
33. Tuiskunen A, Monteil V, Plumet S, Boubis L, Wahlstrom M, Duong V, Buchy P, Lundkvist A, Tolou H, Leparc-Goffart I (2011) Phenotypic and genotypic characterization of dengue virus isolates differentiates dengue fever and dengue hemorrhagic fever from dengue shock syndrome. Arch Virol 156:2023–2032
34. Ubol S, Masrinoul P, Chaijaruwanich J, Kalayanarooj S, Charoensirisuthikul T, Kasisith J (2008) Differences in global gene expression in peripheral blood mononuclear cells indicate a significant role of the innate responses in progression of dengue fever but not dengue hemorrhagic fever. J Infect Dis 197:1459–1467
35. Yauch LE, Zellweger RM, Kotturi MF, Qutubuddin A, Sidney J, Peters B, Prestwood TR, Sette A, Shresta S (2009) A protective role for dengue virus-specific CD8+ T cells. J Immunol 182:4865–4873
36. Yauch LE, Prestwood TR, May MM, Morar MM, Zellweger RM, Peters B, Sette A, Shresta S (2010) CD4+ T cells are not required for the induction of dengue virus-specific CD8+ T cell or antibody responses but contribute to protection after vaccination. J Immunol 185:5405–5416
37. Yoshida T, Saito A, Iwasaki Y, Iijima S, Kurosawa T, Katakai Y, Yasutomi Y, Reimann KA, Hayakawa T, Akari H (2010) Characterization of natural killer cells in tamarins: a technical basis for studies of innate immunity. Front Microbiol 1:128
38. Yoshida T, Omatsu T, Saito A, Katakai Y, Iwasaki Y, Iijima S, Kurosawa T, Hamano M, Nakamura S, Takasaki T, Yasutomi Y, Kurane I, Akari H (2012) CD16(+) natural killer cells play a limited role against primary dengue virus infection in tamarins. Arch Virol 157:363–368
39. Zompi S, Santich BH, Beatty PR, Harris E (2012) Protection from secondary dengue virus infection in a mouse model reveals the role of serotype cross-reactive B and T cells. J Immunol 188:404–416

Journal of Asthma and Allergy

Dovepress
open access to scientific and medical research

Open Access Full Text Article

ORIGINAL RESEARCH

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

Katsuo Karamatsu[1,2]
Kazuhiro Matsuo[3]
Hiroyasu Inada[4]
Yusuke Tsujimura[1]
Yumiko Shiogama[1,2]
Akihiro Matsubara[1,2]
Mitsuo Kawano[5]
Yasuhiro Yasutomi[1,2]

[1]Laboratory of Immunoregulation and Vaccine Research, Tsukuba Primate Research Center, National Institute of Biomedical Innovation, Tsukuba, [2]Division of Immunoregulation, Department of Molecular and Experimental Medicine, Mie University Graduate School of Medicine, Tsu, [3]Department of Research and Development, Japan BCG Laboratory, Tokyo, [4]Department of Pathology, Suzuka University of Medical Science, Suzuka, [5]Department of Microbiology and Molecular Genetics, Mie University Graduate School of Medicine, Tsu, Japan

Correspondence: Yasuhiro Yasutomi
Laboratory of Immunoregulation and Vaccine Research, Tsukuba Primate Research Center, National Institute of Biomedical Innovation, 1-1 Hachimandai, Tsukuba, Ibaraki 305-0843, Japan
Tel +81 29 837 2053
Fax +81 29 837 2053
Email yasutomi@nibio.go.jp

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 antiallergic 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.

submit your manuscript | www.dovepress.com
Dovepress
http://dx.doi.org/10.2147/JAA.S37667

Journal of Asthma and Allergy 2012:5 71–79

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 hematocytometer, 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

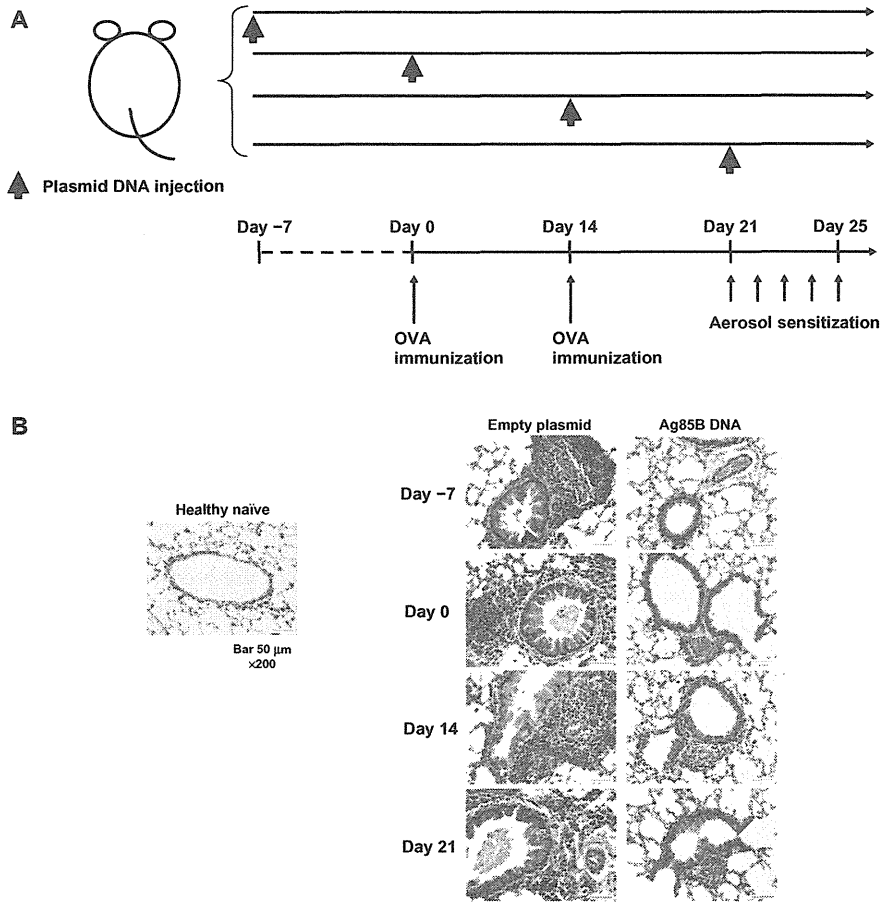

Figure 1 Inhibition of the development of allergic inflammation in lungs by administration of Ag85B DNA vaccine. (**A**) Experimental design used to investigate the effects of Ag85B DNA vaccine on OVA-induced asthma. Mice were subjected to an OVA sensitization scheme,[15] and 50 μg of Ag85B DNA vaccine 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).