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Efficient *in vivo* depletion of CD8$^+$ T lymphocytes in common marmosets by novel CD8 monoclonal antibody administration

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Key words: monoclonal antibody, CD8 T lymphocyte, common marmoset, in vivo depletion

Abstract

In order to directly demonstrate the roles of $CD8^+$ T lymphocytes in non-human primates, *in vivo* depletion of the $CD8^+$ T cells by administration of a CD8-specific monoclonal antibody (mAb) is one of the crucial techniques. Recently, the common marmoset (*Callithrix jacchus*), which is classified as a New World monkey, has been shown useful as an experimental animal model for various human diseases such as multiple sclerosis, Parkinson's disease and a number of infectious diseases. Here we show that an anti-marmoset CD8 mAb 6F10, which we have recently established, efficiently depletes the marmoset $CD8^+$ T lymphocytes *in vivo*, i.e., the administration of 6F10 induces drastic and specific reduction in the ratio of the $CD8^+$ T cell subset for at least three weeks or longer. Our finding will help understand the pivotal role of $CD8^+$ T cells *in vivo* in the control of human diseases.

1. Introduction

The use of non-human primates as experimental animal models is highly effective for research on human diseases. Non-human primates and humans share comparable immune systems as compared with mice and are suitable for the evaluation of innate and adaptive immune responses against several viruses[1,2]. On the other hand, there are also several issues with chimpanzees and macaques. The most prevalent being that the use of the chimpanzee is limited by ethical and financial restrictions [3-6].

A New World monkey, the common marmoset (*Callithrix jacchus*) has several advantages as an experimental animal model. The small size of the marmoset makes it easier to handle and reduces maintenance costs [7]. Recently, it has been reported that the marmoset model is a very useful tool in investigating multiple sclerosis (MS), rheumatoid arthritis (RA) and Parkinson's disease [8-10]. Moreover, the marmoset has an immune system similar to that of humans and is suitable for the evaluation of innate and adaptive immune responses against several viruses which efficiently replicate in the marmoset [11-14].

$CD8^+$ T lymphocytes are a vital component of the adaptive immune response and are crucial to the control and clearance of intracellular pathogens. These cells play critical roles in purging acute infections, limiting persistent infections, and conferring life-long protective immunity. In order to clarify the pivotal role of $CD8^+$ T cells in a variety of non-human primate models for human diseases, *in vivo* depletion of $CD8^+$ T cells by administration of a CD8-specific monoclonal antibody (mAb) is a straightforward technique, although it has been established in Old World monkeys but not in New World monkeys [15-21].

We recently established a novel mAb 6F10 specific for common marmoset

70 CD8 [7]. In this study, we demonstrated for the first time in New World monkeys that
71 the administration of the 6F10 mAb efficiently depleted $CD8^+$ T lymphocytes in
72 marmosets.

2. Materials and Methods

2.1. 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 Primate Research Institute of Kyoto University, Inuyama, Japan. A total of three marmosets, weighing 357-457 g, were used. Common marmosets were caged individually at 27±2 °C in 50±10% humidity with a 12h light-dark cycle (lighting from 7:00 to 19:00) in our facility. All animals were fed twice a day with a standard marmoset diet supplemented with fruit and mealworm. Water was given ad libitum.

2.2. Flow cytometry

Flow cytometry was performed as previously described with a slight modification [22]. A previously established mouse anti-marmoset CD8 mAb named 6F10 was used [7]. The 6F10 mAb was conjugated with allophycocyanin (APC) and by Zenon Mouse IgG labeling Kit (Molecular Probes) according to the manufacturer's instruction. Fifty microliters of whole blood from marmosets was stained with combinations of fluorescence-conjugated mAb: APC-Cy7-conjugated anti-CD3 (SP34-2: Becton Dickinson), PerCP-Cy5.5-conjugated anti-CD4 (L200: BD Pharmingen), PE-conjugated anti-CD8 (CLB-T8/4, 4H8 (CLB hereafter): Sanquin; RPA-T8 (T8 hereafter): Becton Dickinson) and FITC-conjugated anti-CD20 (H299: BECKMAN COULTER). Then, erythrocytes were lysed with FACS lysing solution (Becton Dickinson). After washing with a sample buffer containing phosphate-buffered saline (PBS) and 1% fetal calf serum (FCS), the labeled cells were resuspended in a fix buffer containing PBS and 1% formaldehyde. The expression of the immunolabeled molecules on the lymphocytes was

analyzed with a FACSCanto II flow cytometer (Becton Dickinson). The data analysis was conducted using FlowJo software (Treestar, Inc.).

2.3. *In vitro* binding competition of anti-CD8 mAbs

Fifty microliters of whole blood from marmosets was treated with increasing amounts (1, 10, 100 ng) of the 6F10 mAb on ice for 30 min. After washing with the sample buffer, the cells were stained with fluorescence-conjugated mAbs against CD3, CD4, and CD8 on ice for 30 min. Then, erythrocytes were lysed with FACS lysing solution (Becton Dickinson). After washing with the sample buffer, the labeled cells were resuspended in the fix buffer. The fluorescence intensity of the cells were analyzed at is as described above.

2.4. *In vivo* depletion of $CD8^+$ T lymphocytes

In vivo depletion of the marmoset $CD8^+$ T lymphocytes was performed as previously described [19]. Briefly, the 6F10 or a control mAb (MOPC-21) was administrated subcutaneously to the subject at 10 mg/kg (body weight) followed by intravenous administration at 5 mg/kg in the saphenous vein at a rate of 20 ml/h using a syringe pump on days 3, 7, 10 after the primary administration. The kinetics for the percentages of $CD8^+$ and $CD4^+$ cells in a $CD3^+$ T cell subset as well as the percentages of $CD20^+$ B cells and $CD3^-CD20^-$ cells in the total lymphocytes of each marmoset were periodically monitored during the observation period as indicated.

2.5. Statistical analyses

Statistical analyses of lymphocyte ratios were performed using Student's *t*-test and single-factor ANOVA, followed by Fisher's protected least-significant difference *post hoc* test by using StatView software (SAS Institute, NC, USA).

3. Results

3.1. Lymphocyte subsets in marmosets

We previously demonstrated that the 6F10 mAb specifically detected $CD8^+$ T lymphocytes in marmosets by using flow cytometry as well as immunohistochemical and Western blot analyses [7]. Basic information regarding CD4/CD8 naïve and central/effector memory T cells and NK/NKT cells in marmosets was available from our recent report [13]. We compared the immunoreactivity of the 6F10 mAb with other commercially available CD8 mAbs in lymphocyte subsets of marmosets (Fig. 1). The gating strategy for profiling $CD4^+$ and $CD8^+$ T cells was shown in Figure 1. The percentage of $CD8^+$ T cells in a $CD3^+$ T cell subset as detected by 6F10, CLB or T8 anti-CD8 mAb was comparable (23.5%, 23.2% and 22.9%, respectively). Notably, the 6F10 mAb poorly cross-reacted with tamarin and rhesus macaque $CD8^+$ T cells while CLB and T8 mAbs did with both (data not shown). It is reasonable that the 6F10 mAb showed selected specificity to the marmoset CD8, considering that it was established by immunization with marmoset lymphocytes [7].

3.2. *In vitro* binding competition of anti-CD8 antibodies in $CD8^+$ T cells of marmosets

We initially treated the marmoset lymphocytes with increasing amounts of APC-conjugated 6F10 together with fluorescence-labeled mAbs to CD3 and CD4. It was found that fluorescence intensity for APC on a $CD3^+$ T cell subset was saturated by 10 ng or more of 6F10 mAb (Fig.2A). We then sought to define whether the binding epitope of 6F10 mAb in the marmoset CD8 molecule was overlapped with the epitopes of T8 and CLB mAbs by a competition assay. It was found that fluorescence

intensity in the $CD3^+$ T cell subset treated with the labeled CLB mAb was drastically reduced by the pretreatment of 10 or 100 ng 6F10 (Fig. 2B, C). On the other hand, the 6F10 pretreatment scarcely influenced fluorescence intensity in the cells that reacted with the labeled T8 mAb, irrespective of the amounts of 6F10 (Fig. 2B, C). These results indicated that 6F10 competitively inhibited binding of CLB but not T8 mAbs to CD8, suggesting that the binding epitope for 6F10 was overlapped with that of CLB and was sterically apart from that of T8. In addition, T8 was likely to exhibit greater affinity than CLB to marmoset CD8 (Fig 2B).

3.3. *In vivo* depletion of $CD8^+$ T cells using an anti-marmoset CD8 mAb

We finally examined whether the administration of the 6F10 mAb could influence $CD8^+$ T lymphocytes *in vivo*. Three marmosets were subcutaneously administrated at 10 mg/kg followed by intravenous administration at 5 mg/kg on days 3, 7, 10 after the primary administration. The mAb-treated marmosets did not develop any clinical and hematological signs (data not shown). In order to detect $CD8^+$ T lymphocytes in the 6F10-treated marmosets, we employed T8 mAb, which was found to react with CD8 molecule in the presence of 6F10 as shown in Fig. 2. It was found that at 10 days after the mAb administration the $CD8^+$ T cells were almost completely depleted, followed by gradual recovery to a half of the initial levels at around 4-7 weeks later (Fig. 3). It is noteworthy that the treatment relatively increased in the proportion of $CD4^+$ T cells in compensation for the depletion of $CD8^+$ T cells, while the ratios of $CD20^+$ B cells and $CD3^-CD20^-$ cells were scarcely affected (Fig. 4). In addition, administration of a control antibody (MOPC-21) did not affect any lymphocyte subsets (data not shown). These results demonstrated that the 6F10 mAb was able to specifically deplete $CD8^+$ T cells in marmosets.

4. Discussion

In this study, we attempted to establish a technical basis for the study of $CD8^+$ T cells in marmosets. We assessed the effect of a 6F10 mAb administration *in vivo* and found that $CD8^+$ T cells were efficiently depleted in the blood of the treated monkeys for at least three weeks or longer and that in compensation for the depletion proportion of $CD4^+$ T cells were relatively increased without obvious influence on other lymphocyte subsets such as $CD20^+$ B cells and $CD3^-CD20^-$ cells. This is the first report showing the establishment of new methodology to deplete common marmoset $CD8^+$ T lymphocytes in vivo. Since demand for marmosets as non-human primate models for a variety of inflammatory and autoimmune diseases as well as infectious diseases has been increasing, our findings will provide new techniques to scientists who are eager to examine the pivotal role of $CD8^+$ T lymphocytes in vivo in the onset of the human diseases.

We previously sought to examine the dynamics of cellular immune responses in the acute phase of dengue virus (DENV) infection in a novel marmoset model that we recently developed [12]. We found that the DENV infection in marmosets greatly induced early immune responses of CD4/CD8 central memory T cells, suggesting that the cellular immunity may be associated with the control of primary DENV infection [13]. Considering this, the present techniques to deplete $CD8^+$ T cells *in vivo* will provide a useful tool to further elucidate the functional role of $CD8^+$ T cells in the acute DENV infection.

Common marmosets are suitable for detailed observations of the movement of the extremities and cognitive functions, which approximate those of humans. Therefore, marmosets are highly useful as models of neurological diseases such as Parkinson's, MS and RA. Importantly, the marmoset models for analyzing Parkinson's disease and

196 autoimmune diseases such as MS and RA have already been developed [8-10,23]. $CD8^+$
197 T cells have been implicated in the pathogenesis of autoimmune disorders including
198 diseases of the central nervous system such as MS, encephalomyelitis and diabetes
199 mellitus [24-26]. MS is an immune-mediated disease of the central nervous system
200 leading to demyelination and axonal/neuronal loss. Accumulating evidence points to a
201 key role for $CD8^+$ T cells in the disease; histopathological analyses and compelling
202 observations from animal models indicate that cytotoxic $CD8^+$ T cells target neural cell
203 populations with the potential of causing lesions consistent with MS [27]. RA is a
204 systemic and chronic autoimmune disease characterized mainly by synovial
205 inflammation leading to joint destruction and disability with a huge impact upon the
206 quality of life and life expectancy. Several studies have demonstrated that $CD8^+$ T cells
207 in RA have powerful cytotoxic ability and therefore exhibit the potential to enhance the
208 disease [28]. Thus, our *in vivo* CD8 depletion technics will be valuable in further
209 examining the role of $CD8^+$ T cells in these autoimmune diseases in the marmoset
210 models.

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220 **Figure legends**

221

222 **Figure 1.** Flow cytometric analyses of CD3, CD4 and CD8 expression on lymphocytes
223 in marmosets. Fifty microliters of whole blood specimens from marmosets were
224 stained with APC-Cy7-conjugated anti-CD3, PerCP-Cy5.5-conjugated anti-CD4 and
225 APC-conjugated 6F10 mAbs or PE-conjugated CD8 (CLB or T8) mAb. Then,
226 erythrocytes were lysed and the stained cells were resuspended in the fix buffer.
227 Representative results in a marmoset are shown. The G1 lymphocyte population was
228 selected (left top panel) and a $CD3^+$ T cell subset was gated (right top panel).
229 Fluorescence intensity for CD4 and CD8 in the T cell subset was depicted (lower
230 panels).

231

232 **Figure 2.** Binding competition among anti-CD8 mAbs in marmoset $CD8^+$ T cells. (A)
233 Fifty microliters of whole blood specimens from marmosets were stained with
234 APC-Cy7-conjugated CD3 and PerCP-Cy5.5-conjugated CD4 mAbs and increasing
235 amounts (1, 10, 100 ng) of APC-conjugated 6F10 mAb. Then, erythrocytes were lysed
236 and the stained cells were resuspended in the fix buffer. The fluorescence intensity for
237 $CD4^+$ and $CD8^+$ cells in a $CD3^+$ T cell subset was shown. (B, C) Fifty microliters of
238 whole blood specimens were pretreated with increasing amounts (1, 10, 100 ng) of 6F10
239 mAb, followed by staining with fluorescence-conjugated mAbs against CD3, CD4, and
240 CD8 (CLB or T8). Fluorescence intensity of $CD4^+$ and $CD8^+$ cells in a $CD3^+$ T cell
241 subset was shown (B) and the geometric mean fluorescence of $CD8^+$ T cells labeled by
242 the CLB or T8 mAbs was indicated. We analyzed statistically whether geometric means
243 were different in each antibody by using StatView software.

Figure 3. *In vivo* depletion of $CD8^+$ T cells by administration of a marmoset anti-CD8 mAb 6F10. Periodical kinetics for the fluorescence intensity of $CD4^+$ and $CD8^+$ cells in a $CD3^+$ T cell subset of Cj175 administered subcutaneously with 10 mg/kg of the 6F10 mAb, followed by 5 mg/kg administration intravenously at days 3, 7, and 10 were shown.

Figure 4. The kinetics for the ratios of $CD8^+$ cells (A) and $CD4^+$ cells (B) in a $CD3^+$ T cell subset as well as $CD20^+$ B cells (C) and $CD3^-CD20^-$ cells (D) in total lymphocytes of each marmoset after the administration of the 6F10 mAb were shown.

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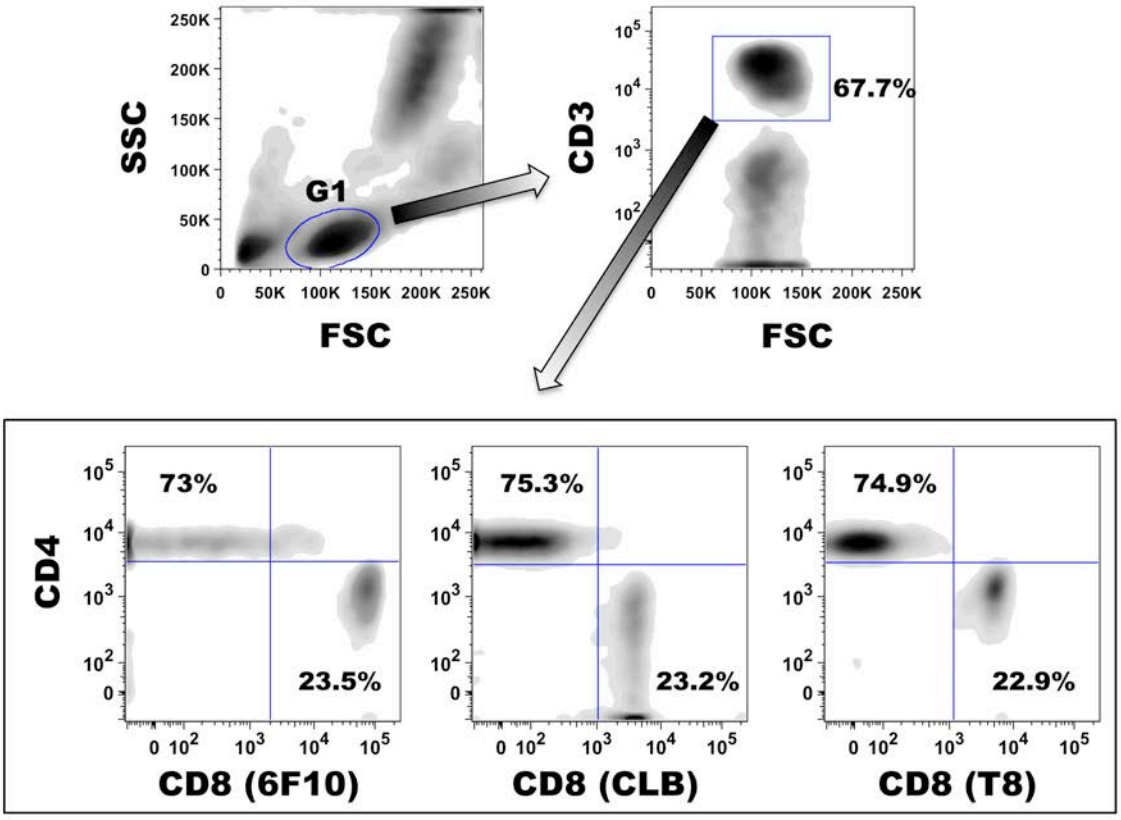
Conflict of Interest Statement:

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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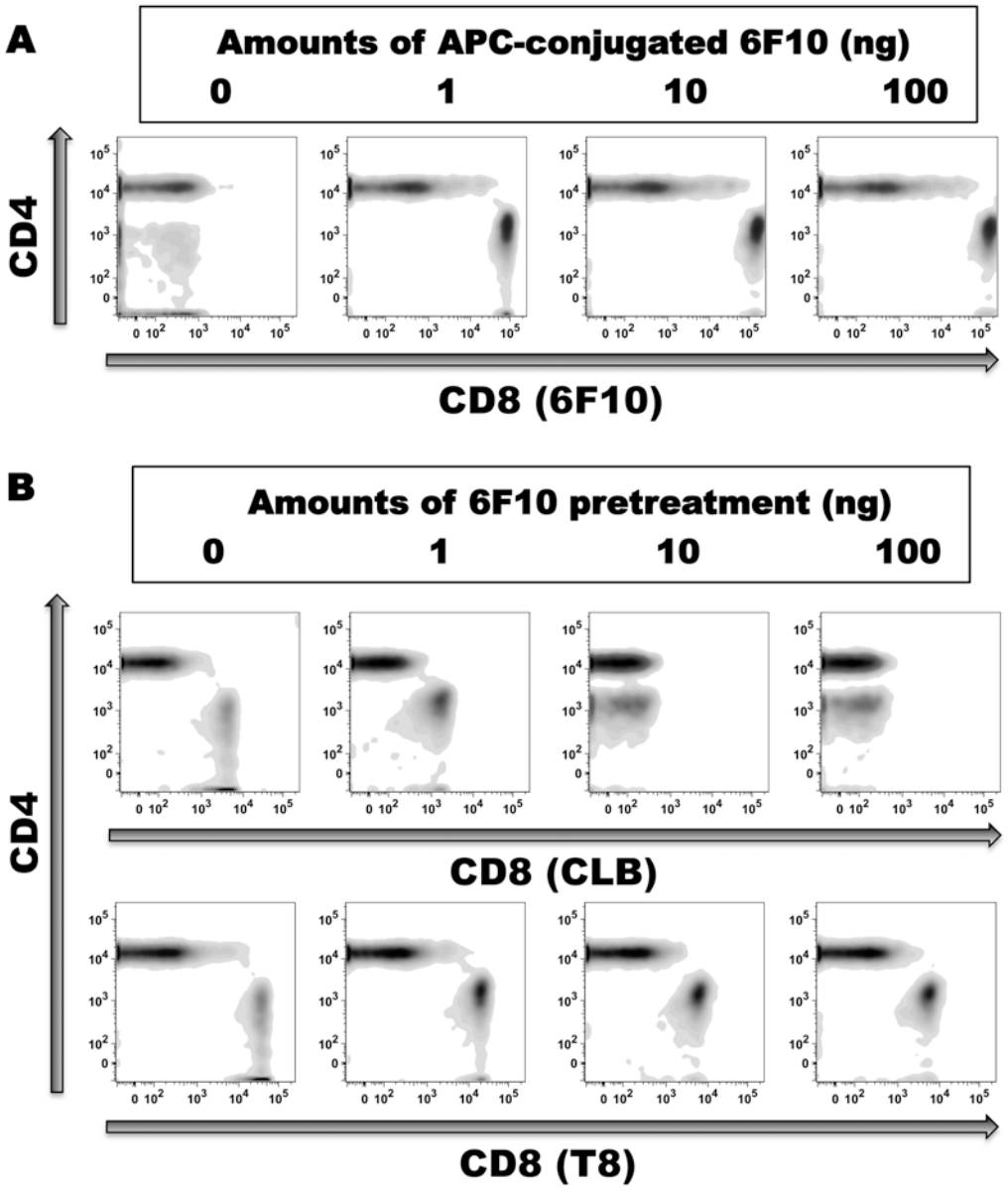
CD8 (CLB)
CD8 (T8)

345

Fig. 2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CD4
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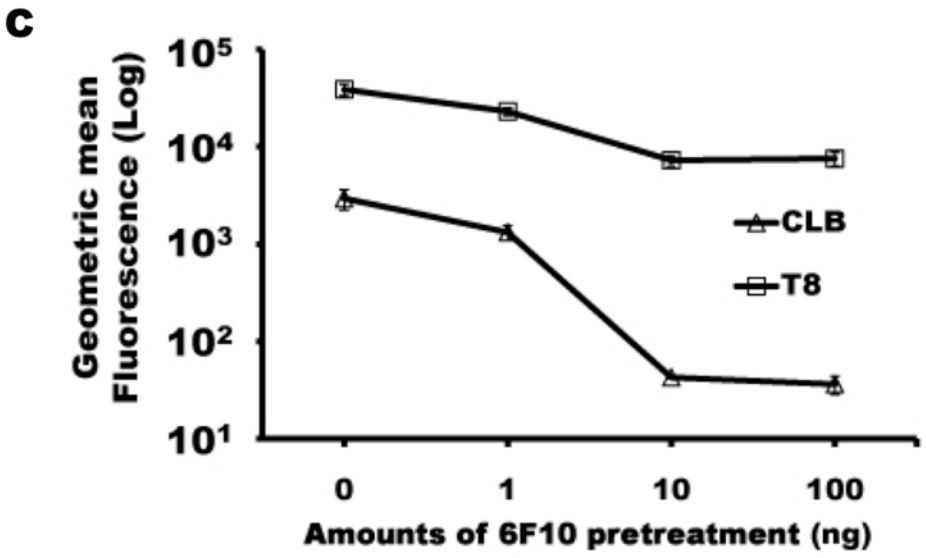
CD8 (T8)

346

Fig. 2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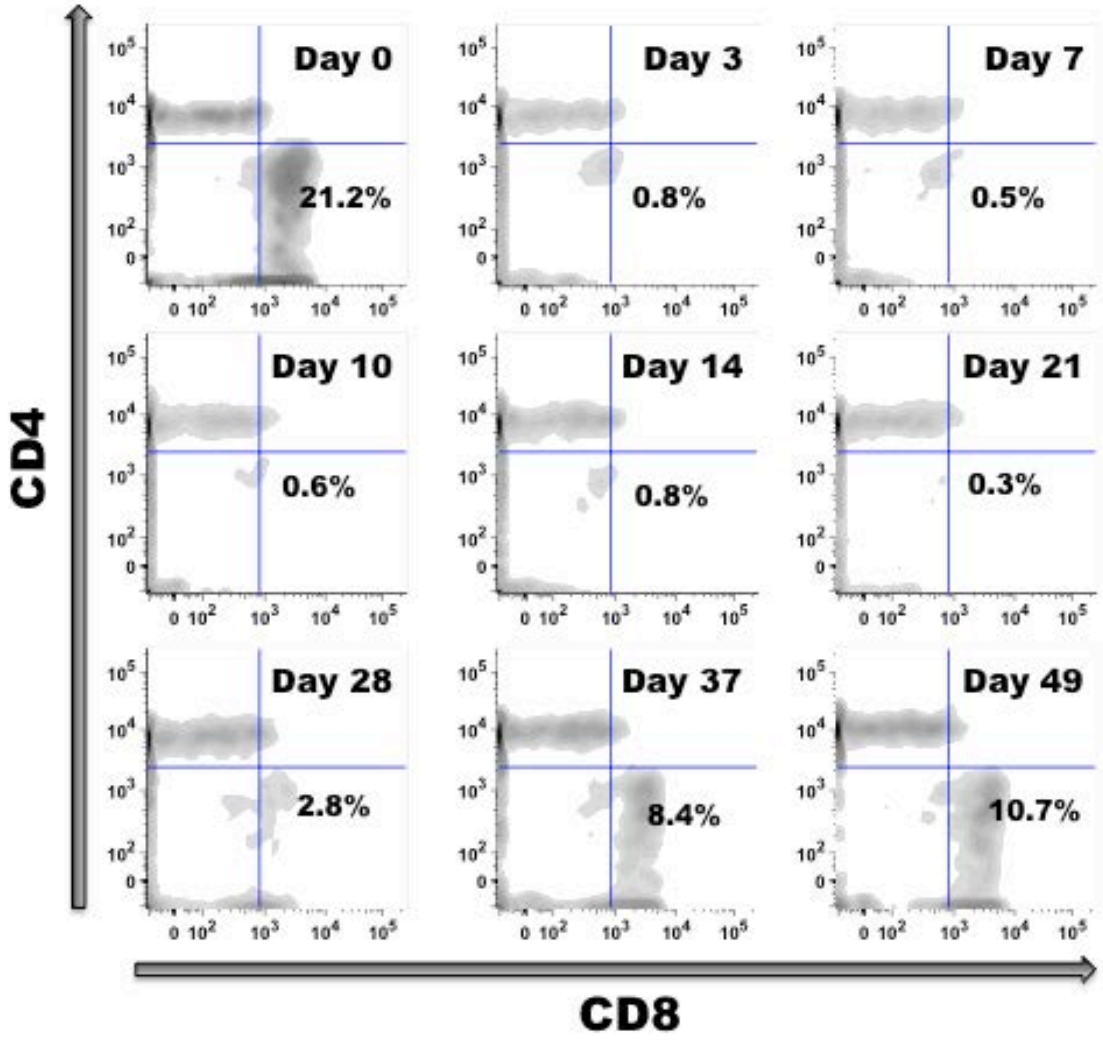
347

348

Fig. 3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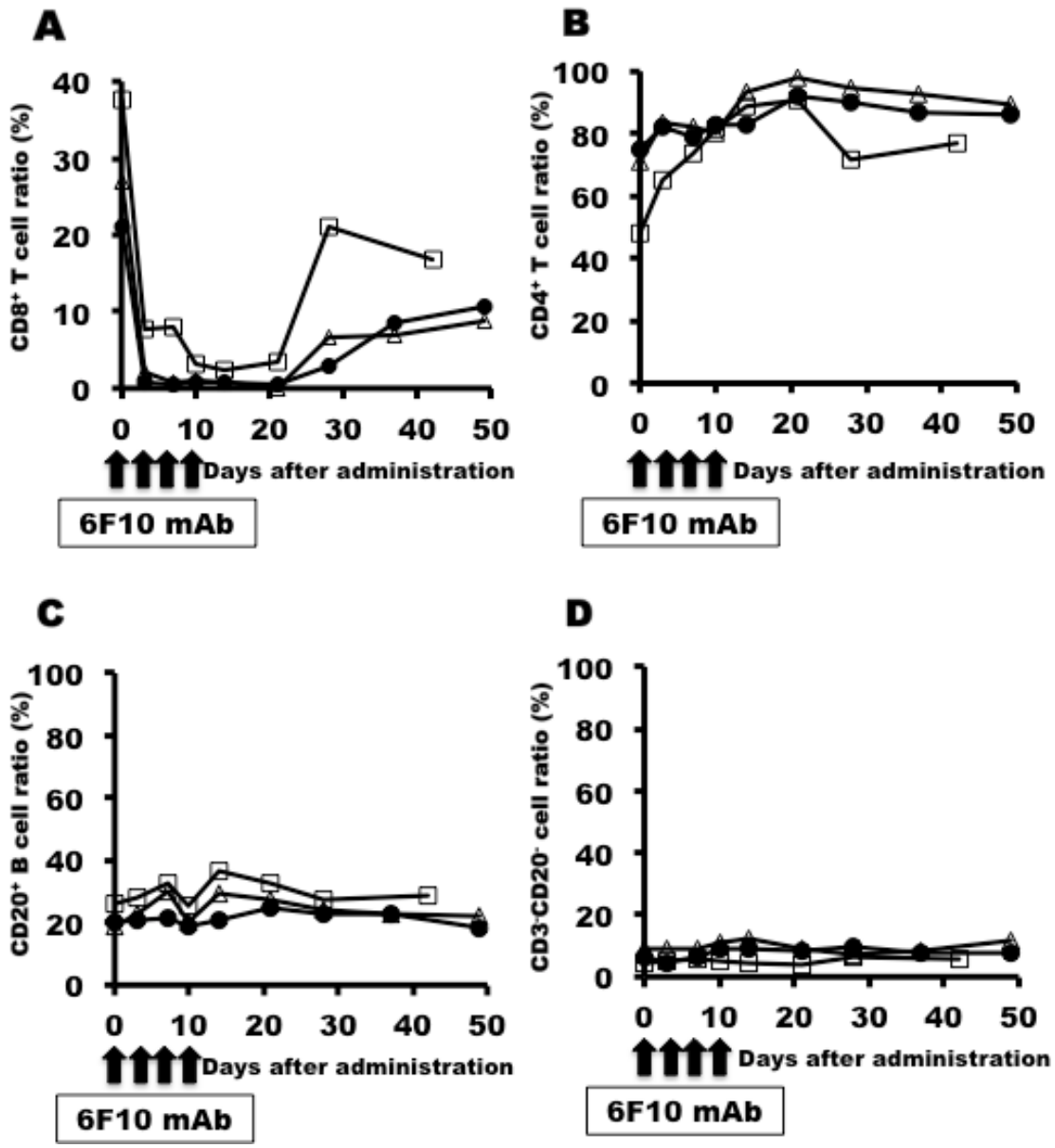
CD4
CD8

352 **Fig. 4**

353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Days after administration
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D
CD3-CD20- cell ratio (%)
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