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3 **Efficient *in vivo* depletion of CD8^{+} T lymphocytes in common marmosets by novel**
4 **CD8 monoclonal antibody administration**

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

28

29    **Abstract**

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31    In order to directly demonstrate the roles of $CD8^+$ T lymphocytes in non-human

32    primates, *in vivo* depletion of the $CD8^+$ T cells by administration of a CD8-specific

33    monoclonal antibody (mAb) is one of the crucial techniques. Recently, the common

34    marmoset (*Callithrix jacchus*), which is classified as a New World monkey, has been

35    shown useful as an experimental animal model for various human diseases such as

36    multiple sclerosis, Parkinson's disease and a number of infectious diseases. Here we

37    show that an anti-marmoset CD8 mAb 6F10, which we have recently established,

38    efficiently depletes the marmoset $CD8^+$ T lymphocytes *in vivo*, i.e., the administration

39    of 6F10 induces drastic and specific reduction in the ratio of the $CD8^+$ T cell subset for

40    at least three weeks or longer. Our finding will help understand the pivotal role of

41    $CD8^+$ T cells *in vivo* in the control of human diseases.

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## 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.

73  **2. Materials and Methods**

74  **2.1. Animals**

75      All animal studies were conducted in accordance with the protocols of

76  experimental procedures that were approved by the Animal Welfare and Animal Care

77  Committee of the Primate Research Institute of Kyoto University, Inuyama, Japan. A

78  total of three marmosets, weighing 357-457 g, were used. Common marmosets were

79  caged individually at 27±2 °C in 50±10% humidity with a 12h light-dark cycle

80  (lighting from 7:00 to 19:00) in our facility. All animals were fed twice a day with a

81  standard marmoset diet supplemented with fruit and mealworm. Water was given ad

82  libitum.

83  **2.2. Flow cytometry**

84      Flow cytometry was performed as previously described with a slight modification

85  [22]. A previously established mouse anti-marmoset CD8 mAb named 6F10 was used

86  [7]. The 6F10 mAb was conjugated with allophycocyanin (APC) and by Zenon Mouse

87  IgG labeling Kit (Molecular Probes) according to the manufacturer's instruction. Fifty

88  microliters of whole blood from marmosets was stained with combinations of

89  fluorescence-conjugated mAb: APC-Cy7-conjugated anti-CD3 (SP34-2: Becton

90  Dickinson), PerCP-Cy5.5-conjugated anti-CD4 (L200: BD Pharmingen), PE-conjugated

91  anti-CD8 (CLB-T8/4, 4H8 (CLB hereafter): Sanquin; RPA-T8 (T8 hereafter): Becton

92  Dickinson) and FITC-conjugated anti-CD20 (H299: BECKMAN COULTER). Then,

93  erythrocytes were lysed with FACS lysing solution (Becton Dickinson). After washing

94  with a sample buffer containing phosphate-buffered saline (PBS) and 1% fetal calf

95  serum (FCS), the labeled cells were resuspended in a fix buffer containing PBS and 1%

96  formaldehyde. The expression of the immunolabeled molecules on the lymphocytes was

97 analyzed with a FACSCanto II flow cytometer (Becton Dickinson). The data analysis

98 was conducted using FlowJo software (Treestar, Inc.).

99 **2.3. *In vitro* binding competition of anti-CD8 mAbs**

100 Fifty microliters of whole blood from marmosets was treated with increasing

101 amounts (1, 10, 100 ng) of the 6F10 mAb on ice for 30 min. After washing with the

102 sample buffer, the cells were stained with fluorescence-conjugated mAbs against CD3,

103 CD4, and CD8 on ice for 30 min. Then, erythrocytes were lysed with FACS lysing

104 solution (Becton Dickinson). After washing with the sample buffer, the labeled cells

105 were resuspended in the fix buffer. The fluorescence intensity of the cells were analyzed

106 at is as described above.

107 **2.4. *In vivo* depletion of CD8$^+$ T lymphocytes**

108 *In vivo* depletion of the marmoset CD8$^+$ T lymphocytes was performed as

109 previously described [19]. Briefly, the 6F10 or a control mAb (MOPC-21) was

110 administrated subcutaneously to the subject at 10 mg/kg (body weight) followed by

111 intravenous administration at 5 mg/kg in the saphenous vein at a rate of 20 ml/h using

112 a syringe pump on days 3, 7, 10 after the primary administration. The kinetics for the

113 percentages of CD8$^+$ and CD4$^+$ cells in a CD3$^+$ T cell subset as well as the percentages

114 of CD20$^+$ B cells and CD3$^-$CD20$^-$ cells in the total lymphocytes of each marmoset were

115 periodically monitored during the observation period as indicated.

116 **2.5.** Statistical analyses

117 Statistical analyses of lymphocyte ratios were performed using

118 Student's *t*-test and single-factor ANOVA, followed by Fisher's protected

119 least-significant difference *post hoc* test by using StatView software (SAS

120 Institute, NC, USA).

121   **3. Results**

122

123   **3.1. Lymphocyte subsets in marmosets**

124        We previously demonstrated that the 6F10 mAb specifically detected CD8^{+}

125   T lymphocytes in marmosets by using flow cytometry as well as immunohistochemical

126   and Western blot analyses [7]. Basic information regarding CD4/CD8 naïve and

127   central/effector memory T cells and NK/NKT cells in marmosets was available from

128   our recent report [13]. We compared the immunoreactivity of the 6F10 mAb with other

129   commercially available CD8 mAbs in lymphocyte subsets of marmosets (Fig. 1). The

130   gating strategy for profiling CD4^{+} and CD8^{+} T cells was shown in Figure 1. The

131   percentage of CD8^{+} T cells in a CD3^{+} T cell subset as detected by 6F10, CLB or T8

132   anti-CD8 mAb was comparable (23.5%, 23.2% and 22.9%, respectively). Notably, the

133   6F10 mAb poorly cross-reacted with tamarin and rhesus macaque CD8^{+} T cells while

134   CLB and T8 mAbs did with both (data not shown). It is reasonable that the 6F10 mAb

135   showed selected specificity to the marmoset CD8, considering that it was established

136   by immunization with marmoset lymphocytes [7].

137

138   **3.2. *In vitro* binding competition of anti-CD8 antibodies in CD8^{+} T cells of**

139   **marmosets**

140        We initially treated the marmoset lymphocytes with increasing amounts of

141   APC-conjugated 6F10 together with fluorescence-labeled mAbs to CD3 and CD4. It

142   was found that fluorescence intensity for APC on a CD3^{+} T cell subset was saturated

143   by 10 ng or more of 6F10 mAb (Fig.2A). We then sought to define whether the

144   binding epitope of 6F10 mAb in the marmoset CD8 molecule was overlapped with the

145   epitopes of T8 and CLB mAbs by a competition assay. It was found that fluorescence

146 intensity in the CD3$^+$ T cell subset treated with the labeled CLB mAb was drastically

147 reduced by the pretreatment of 10 or 100 ng 6F10 (Fig. 2B, C). On the other hand, the

148 6F10 pretreatment scarcely influenced fluorescence intensity in the cells that reacted

149 with the labeled T8 mAb, irrespective of the amounts of 6F10 (Fig. 2B, C). These

150 results indicated that 6F10 competitively inhibited binding of CLB but not T8 mAbs to

151 CD8, suggesting that the binding epitope for 6F10 was overlapped with that of CLB

152 and was sterically apart from that of T8. In addition, T8 was likely to exhibit greater

153 affinity than CLB to marmoset CD8 (Fig 2B).

154

155 **3.3. *In vivo* depletion of CD8$^+$ T cells using an anti-marmoset CD8 mAb**

156 We finally examined whether the administration of the 6F10 mAb could influence

157 CD8$^+$ T lymphocytes *in vivo*. Three marmosets were subcutaneously administrated at

158 10 mg/kg followed by intravenous administration at 5 mg/kg on days 3, 7, 10 after the

159 primary administration. The mAb-treated marmosets did not develop any clinical and

160 hematological signs (data not shown). In order to detect CD8$^+$ T lymphocytes in the

161 6F10-treated marmosets, we employed T8 mAb, which was found to react with CD8

162 molecule in the presence of 6F10 as shown in Fig. 2. It was found that at 10 days after

163 the mAb administration the CD8$^+$ T cells were almost completely depleted, followed

164 by gradual recovery to a half of the initial levels at around 4-7 weeks later (Fig. 3). It is

165 noteworthy that the treatment relatively increased in the proportion of CD4$^+$ T cells in

166 compensation for the depletion of CD8$^+$ T cells, while the ratios of CD20$^+$ B cells and

167 CD3$^-$CD20$^-$ cells were scarcely affected (Fig. 4). In addition, administration of a

168 control antibody (MOPC-21) did not affect any lymphocyte subsets (data not shown).

169 These results demonstrated that the 6F10 mAb was able to specifically deplete CD8$^+$ T

170 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.

219

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.

244    **Figure 3.** *In vivo* depletion of CD8$^+$ T cells by administration of a marmoset anti-CD8

245    mAb 6F10. Periodical kinetics for the fluorescence intensity of CD4$^+$ and CD8$^+$ cells

246    in a CD3$^+$ T cell subset of Cj175 administered subcutaneously with 10 mg/kg of the

247    6F10 mAb, followed by 5 mg/kg administration intravenously at days 3, 7, and 10

248    were shown.

249

250    **Figure 4.** The kinetics for the ratios of CD8$^+$ cells (A) and CD4$^+$ cells (B) in a CD3$^+$ T

251    cell subset as well as CD20$^+$ B cells (C) and CD3$^-$CD20$^-$ cells (D) in total lymphocytes

252    of each marmoset after the administration of the 6F10 mAb were shown.

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341    **Conflict of Interest Statement:**

342    The authors declare that the research was conducted in the absence of any commercial

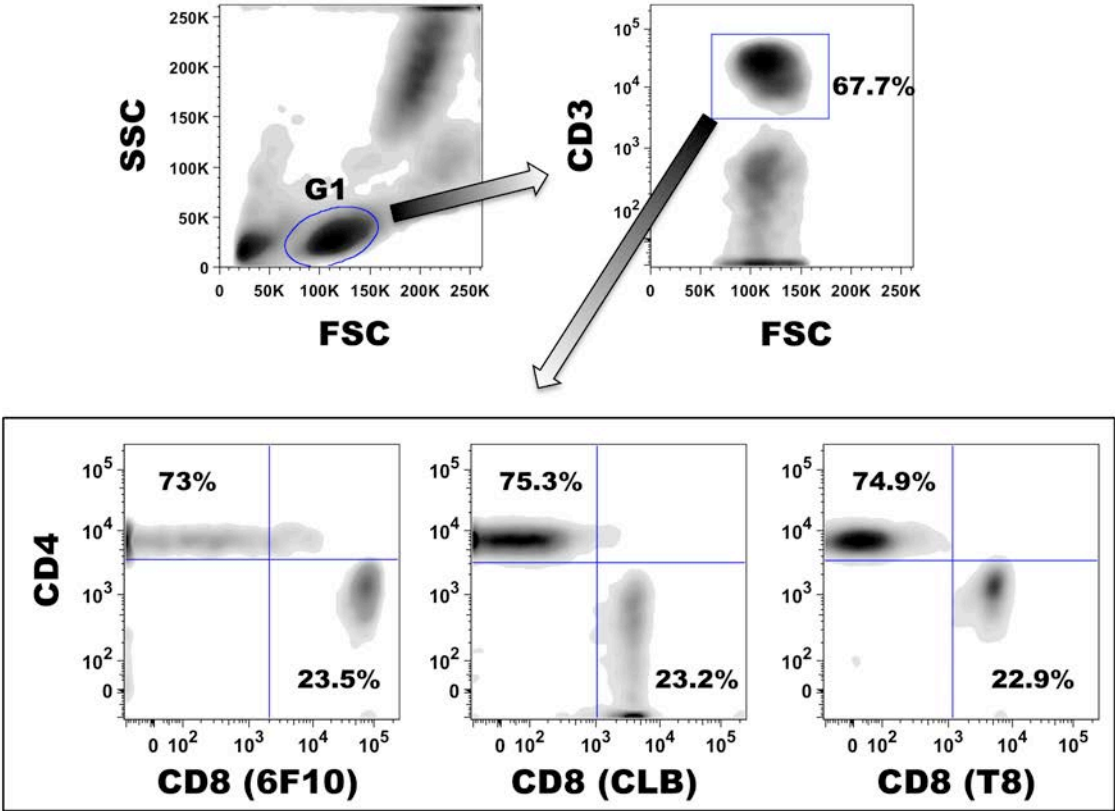343    or financial relationships that could be construed as a potential conflict of interest.
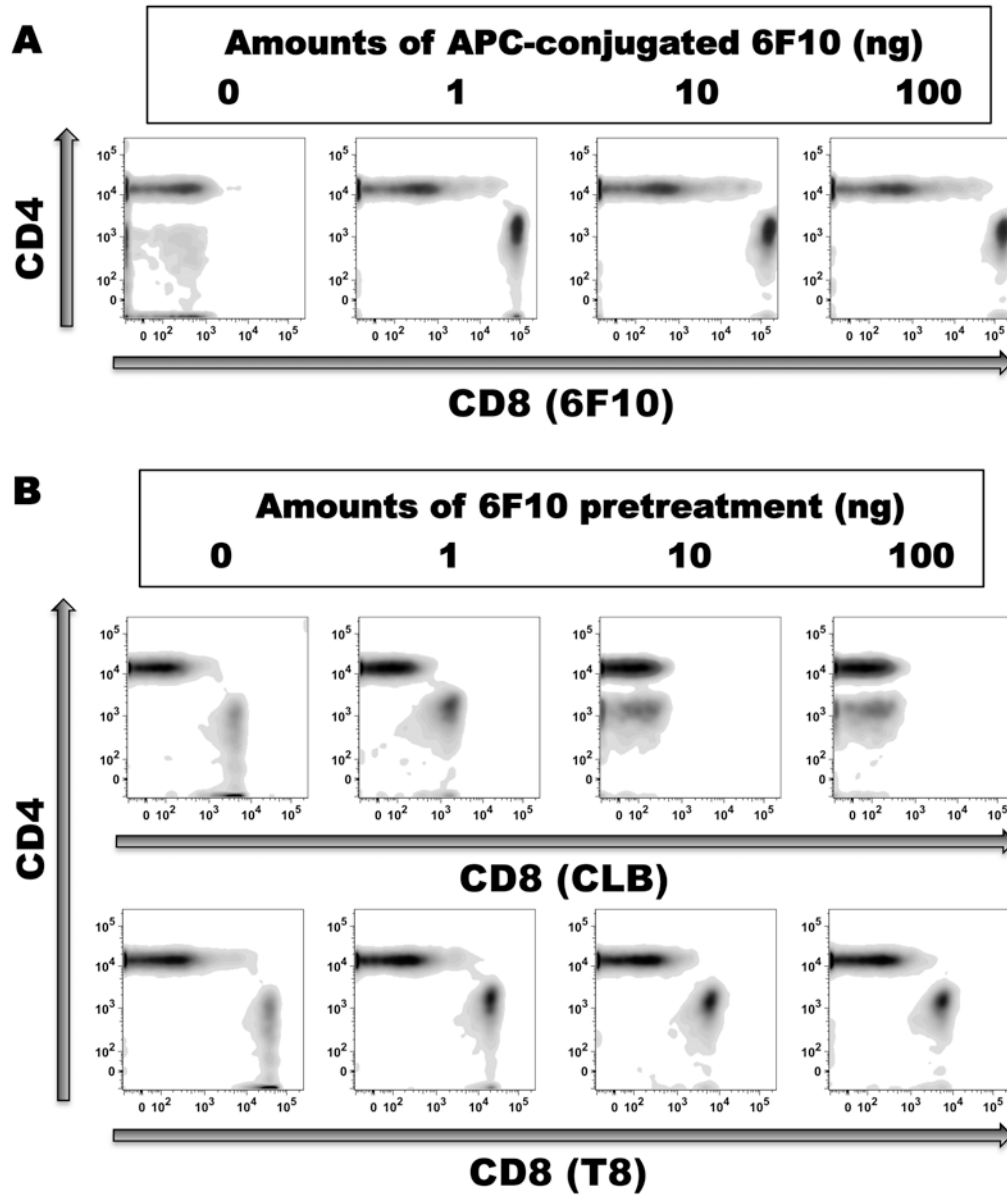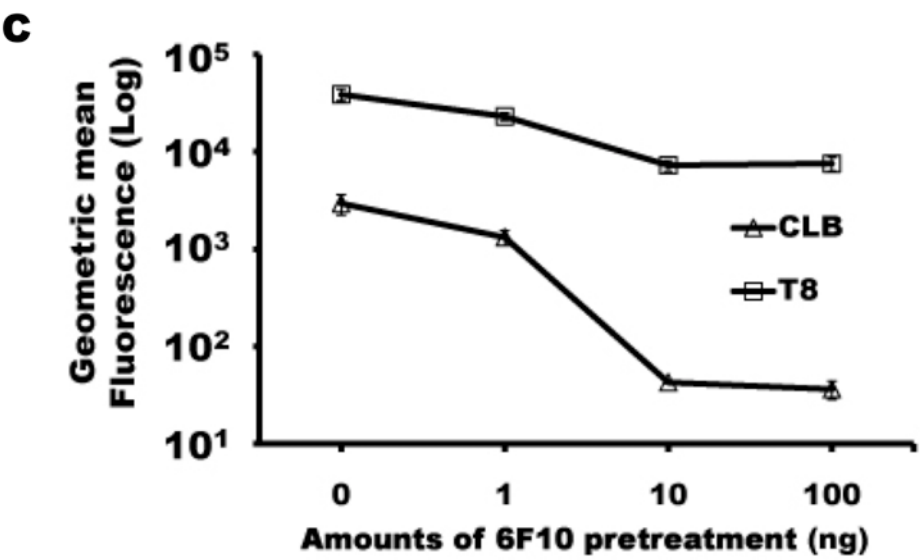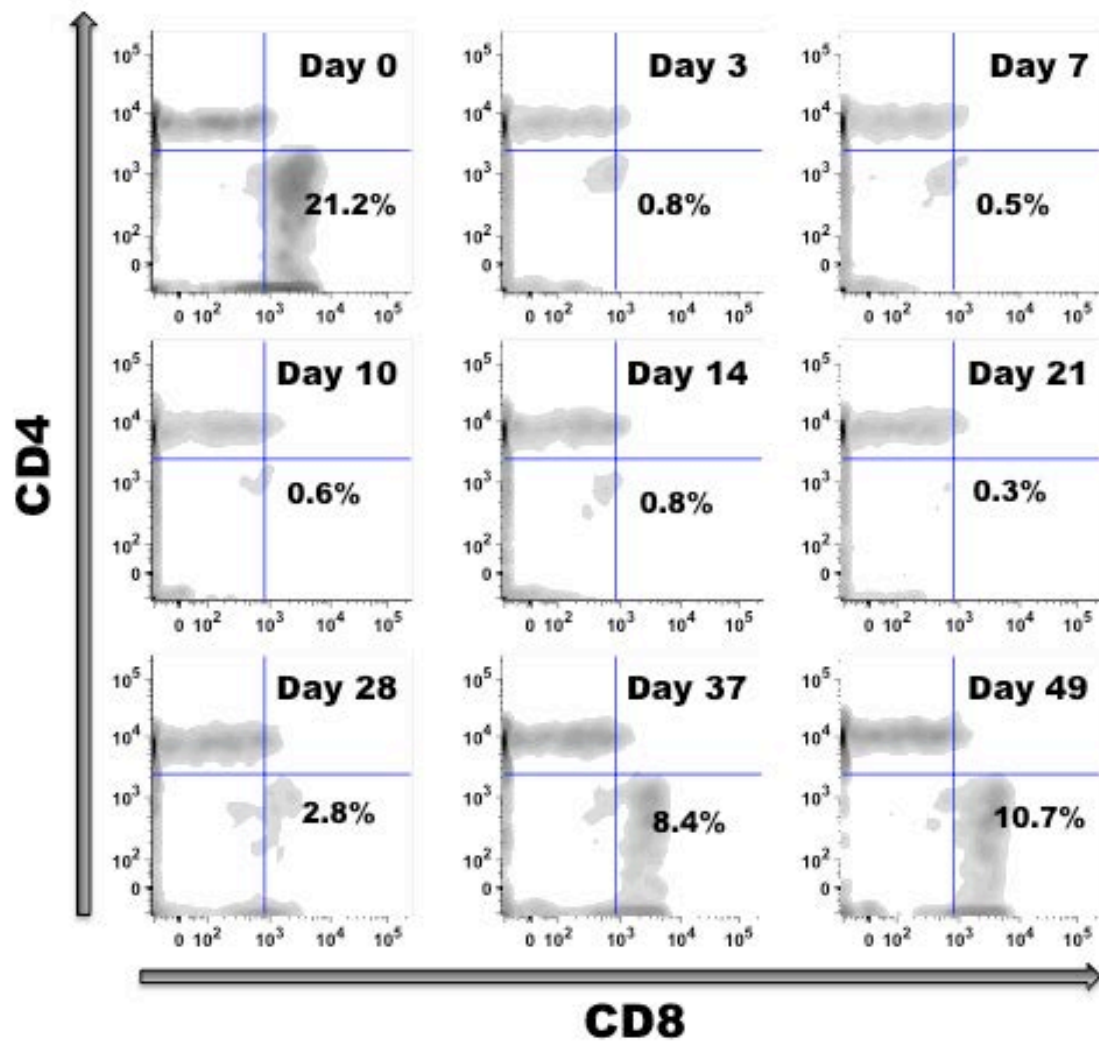
344

Fig. 1



345

**Fig. 2**

**A**

Amounts of APC-conjugated 6F10 (ng)

CD4 / CD8 (6F10)

**B**

Amounts of 6F10 pretreatment (ng)

CD4 / CD8 (CLB)

CD4 / CD8 (T8)

346

18

Fig. 2

c



347
348

**Fig. 3**

**Fig. 4**