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The protective effect of CD40 ligand–CD40 signalling is limited during the early phase of *Plasmodium* infection

Shin-Ichi Inoue [a], Mamoru Niikura [a], Megumi Inoue [a], Shoichiro Mineo [a], Yasushi Kawakami [c], Akihiko Uchida [d], Hiroaki Ohnishi [b], Shigeru Kamiya [a], Takashi Watanabe [b], Fumie Kobayashi [a,*]

[a] *Departments of Infectious Diseases, Kyorin University School of Medicine, 20-2, Shinkawa 6, Mitaka-city, Tokyo 181-8611, Japan*
[b] *Laboratory Medicine, Kyorin University School of Medicine, 20-2, Shinkawa 6, Mitaka-city, Tokyo 181-8611, Japan*
[c] *Laboratory of Parasitology, School of Life and Environmental Science, Azabu University, 17-71, Fuchinobe 1, Sagamihara-city, Kanagawa 252-0206, Japan*
[d] *Yamazaki Gakuen University, 7-2, Minami-osawa 4, Hachiohji-city, Tokyo 192-0364, Japan*

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ABSTRACT

γδ T cells are essential for eliminating *Plasmodium berghei* XAT. Because administration of the agonistic anti-CD40 antibody can induce elimination of *P. berghei* XAT parasites in γδ T cell-deficient mice, we considered that γδ T cells might activate dendritic cells via CD40 signalling during infection. Here we report that administration of the anti-CD40 antibody to γδ T cell-deficient mice 3–10 days post-*P. berghei* XAT infection could eliminate the parasites. Our data suggest that dendritic cell activation via γδ T cells expressing CD40 ligand is critical during the early phase of infection.

1. Introduction

Malaria is one of the most serious public health problems in the world, particularly in tropical and subtropical areas. *Plasmodium* is a malaria-causing protozoan parasite. Previous studies have shown that dendritic cells (DCs) phagocytise blood-stage *Plasmodium* for processing and that DCs present malaria antigens to $CD4^+$ αβ T cells [1–7]. DCs play fundamental roles in the initiation of immune responses against pathogens. Because MHC class II deficient mice do not control blood-stage *Plasmodium yoelii* and *Plasmodium chabaudi* parasites, this conventional pathway also functions in *Plasmodium* infection [8]. There are several DC subpopulations in spleen, which is the central organ for protective immunity against *Plasmodium* infection [9–12]. Conventional DCs (cDCs), whose phenotype is $CD11c^+$ $B220^-$, produce IL-12 to strongly induce the Th1 response. Therefore, cDCs are thought to be key players for initiation of the immune response that eliminates *Plasmodium* parasites [13].

As innate T lymphocytes, γδ T cells are the first line of defence against infectious pathogens. It has been reported that γδ T cells play critical roles in protective immunity against various infectious diseases, including those associated with *Leishmania major*, *Toxoplasma gondii*, *Listeria monocytogenes*, and West Nile virus [14–17]. In patients with malaria, the number of γδ T cells increases in the peripheral blood and spleen [18]. Moreover, γδ T cells can recognise malaria antigens and are activated after *in vitro* culture with *Plasmodium falciparum*-infected red blood cells [19]. Using a rodent model of malaria, we recently showed that γδ T cell-deficient mice (TCR-δ KO mice) could not eliminate the non-lethal malaria parasite strain *Plasmodium berghei* XAT [13]. Moreover, several other groups reported that γδ T cells have protective effects on blood-stage *P. falciparum* and *P. chabaudi* parasites [20,21]. Our previous study found that γδ T cells begin to produce IFN-γ and express CD40 ligand (CD40L) on the cell surface in the early phase of *P. berghei* XAT infection. It is generally assumed that CD40L is expressed on activated $CD4^+$ αβ T cells to accelerate DC activation by CD40L–CD40 signalling [13,22]. The agonistic anti-CD40 monoclonal antibody (mAb) is thought to stimulate DCs via CD40 on the surface of DCs in the presence of antigens [23]. Because administration of agonistic anti-CD40 mAb controls *P. berghei* XAT parasites even in TCR-δ KO mice, CD40L expression on γδ T cells could boost DC activation. However, it remains to be determined whether CD40L–CD40 signalling plays a critical role in DC activation during malaria infection. Here we show that

* Corresponding author. Fax: +81 422 44 4603.
E-mail address: fumfum@ks.kyorin-u.ac.jp (F. Kobayashi).

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administration of the anti-CD40 mAb induces DC activation. Furthermore, we report that the timing of anti-CD40 mAb treatment in TCR-δ KO mice is limited to the early phase of *P. berghei* XAT infection.

2. Materials and methods

2.1. Mice

C57BL/6J wild-type (WT) mice (CREA Japan) and TCR-δ KO mice (The Jackson Laboratories) were bred in the pathogen-free unit of the animal facility of Kyorin University. All animals were female and 8–12 weeks of age at the time of infection. The Kyorin University School of Medicine Animal Care Committee approved all animal protocols.

2.2. Parasites and infection

An attenuated derivative of *P. berghei* [24], *P. berghei* XAT, was used as described in previous studies [13,25,26]. The parasites were stored as frozen stocks in liquid nitrogen. Freshly thawed parasites were passaged once through naïve WT mice, and 10^4 infected red blood cells (iRBCs) from passaged mice were intravenously injected into experimental mice. The resulting parasitemia was assessed by counting 250–10000 RBCs in a Giemsa-stained thin blood film. The percentage of parasitemia was calculated as follows: [(number of iRBCs)/(total number of RBCs)] × 100.

2.3. Flow cytometry

Each day post-infection (p.i.), single spleen cell suspensions from WT mice and TCR-δ KO mice were stained using the fluorescent antibody method in cold PBS containing 0.5% BSA (Sigma) and 0.01% sodium azide (staining buffer) for FACS analyses. Fluorescein isothiocyanate (FITC)-conjugated anti-MHC-II, anti-CD40, anti-CD80, and anti-CD86 mAbs, phycoerythrin (PE)-conjugated anti-B220 mAb, allophycocyanin (APC)-conjugated anti-CD3ε and anti-CD19 mAbs, as well as APC/Cy7-conjugated anti-CD11c mAb were used for DC analyses. PE-conjugated anti-CD40L (CD154) mAb, APC-conjugated anti-CD3ε mAb, PE/Cy7-conjugated anti-TCR-γδ mAb, APC/Cy7 anti-CD4 mAb, and Pacific Blue-conjugated anti-TCR-β mAb were used to analyse γδ T cells and $CD4^+$ T cells. All mAbs were purchased from BioLegend Japan. Analyses were performed using a FACS Canto II with FACS Diva software (BD Biosciences). Data were analysed using FlowJo software (Tree Star).

2.4. Administration of the anti-CD40 agonistic mAb into Plasmodium-infected mice

The anti-CD40 mAb (clone 1C10; BioLegend) was intravenously injected (100 μg per mouse) into *P. berghei* XAT-infected mice once on days 0, 3, 4, 5, 7, 10, 14 or 17 p.i. Normal rat immunoglobulin G (IgG) (Cappel Research Products) was used as a control for the anti-CD40 mAb.

2.5. Statistical methods

Statistical analyses were performed using Student's *t* tests and Statcel (OMS Ltd).

3. Results

3.1. Enhanced dendritic cell activation by the anti-CD40 mAb

We previously showed that γδ T cells play critical roles in protective immunity against blood-stage *P. berghei* XAT parasites, a non-lethal strain in control wild-type (WT) mice [13,25]. Although TCR-δ KO mice could not eliminate *P. berghei* XAT parasites, administration of the agonistic anti-CD40 mAb led to elimination of the parasites in the absence of γδ T cells [13]. We therefore postulated that the agonistic anti-CD40 mAb would contribute to activation of splenic conventional DCs, thereby eliminating parasites even in TCR-δ KO mice. However, it is unclear whether administration of the agonistic anti-CD40 mAb can actually enhance DC activation in mice. DCs were transiently activated on day 5 p.i. with *P. berghei* XAT parasites [13]. Therefore, we inoculated *P. berghei* XAT-infected red blood cells (iRBCs) into WT and TCR-δ KO mice and injected the agonistic anti-CD40 mAb or control IgG on day 4 p.i. On day 5 p.i., we compared the expression levels of activation markers, such as MHC class II (MHC-II), CD40, CD80, and CD86 on the surface of splenic DCs from each group of mice (Fig. 1A–H). Expression of the markers on DCs from *P. berghei* XAT-infected WT mice administered control IgG significantly increased compared with DCs from naïve WT mice, confirming our previous report [13]. In mice administered control IgG, MHC-II, CD40, and CD80 expression on DCs from TCR-δ KO mice was significantly reduced by day 5 p.i compared to DCs from WT mice (Fig. 1A–H). Administration of the anti-CD40 mAb to WT mice upregulated DC expression of CD40, CD80, and CD86, but not of MHC-II, on day 5 p.i. In contrast, administration of the anti-CD40 mAb into TCR-δ KO mice upregulated expression of these four markers on DCs on day 5 p.i. However, CD86 expression on day 5 p.i. in TCR-δ KO mice was statistically lower compared with WT mice (Fig. 1A–H). These results suggest that the anti-CD40 mAb enhances DC activation in mice after *Plasmodium* infection.

Previous studies showed that *Plasmodium* infection leads to DC expansion in the spleen [13,27,28]. Therefore, we estimated splenic DC numbers in WT and TCR-δ KO mice and compared the effects of the anti-CD40 mAb on DC expansion. In *P. berghei* XAT-infected WT mice on day 5 p.i., administration of the anti-CD40 mAb significantly reduced splenic DC numbers compared with control IgG (Fig. 1I). However, splenic DC numbers in *P. berghei* XAT-infected TCR-δ KO mice administered the anti-CD40 mAb were similar to those in mice administered control IgG (Fig. 1I). These results suggest that administration of the anti-CD40 mAb to *P. berghei* XAT-infected WT mice induces excessive DC activation and apoptosis [29,30].

3.2. CD40L expression on γδ T cells and $CD4^+$T cells in spleen

Our previous study showed that γδ T cells express CD40L on day 5 after *P. berghei* XAT infection. However, in the previous study, we did not investigate whether CD40L expression on γδ T cells and $CD4^+$T cells in spleen change during infection. Therefore, we examined CD40L expression on γδ T cells and $CD4^+$T cells in spleens from naïve mice (day 0 p.i.) and from *P. berghei* XAT-infected mice on days 5, 7, 9, 11, and 14 p.i. (Fig. 2). γδ T cells began to express CD40L on their surface day 5 p.i. In contrast, $CD4^+$ T cells increased in CD40L expression starting day 7 p.i. in both WT and TCR-δ KO mice. The proportion of CD40L-expressing γδ T cells was slightly decreased on days 9–11 p.i. Moreover, the proportion of CD40L-expressing $CD4^+$ T cells in WT mice was comparable to that in TCR-δ KO mice from days 7 to 14 p.i. These results suggest that CD40L is continuously expressed on γδ T cells and $CD4^+$ T cells during *Plasmodium* infection.

3.3. CD40 signalling is required to control Plasmodium parasites during the early phase of infection

In our previous report, we showed using immunodepletion of γδ T cells that γδ T cells function prior to day 9 after *P. berghei* XAT infection [13]. However, the most effective timing of agonistic

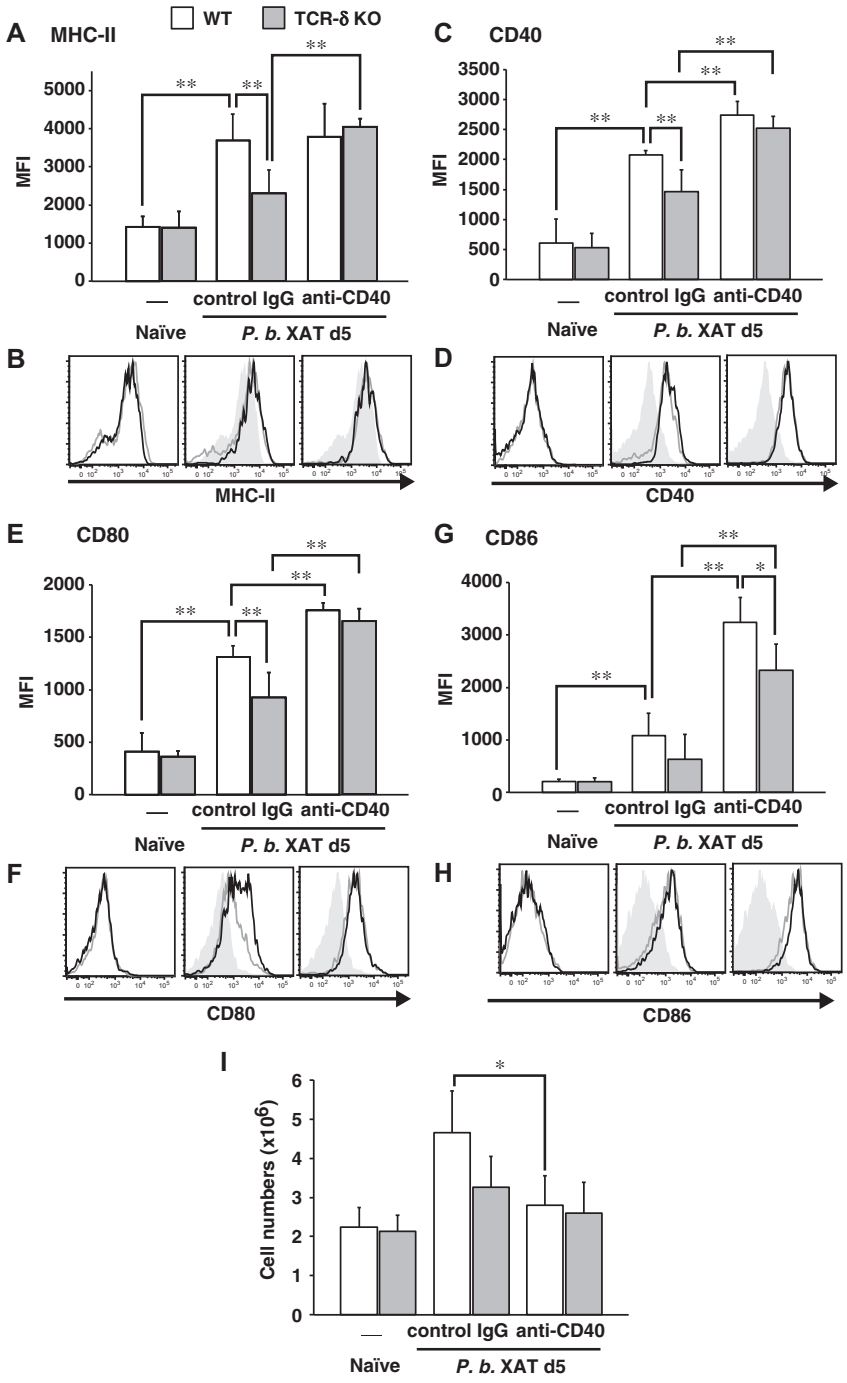

Fig. 1. The agonistic anti-CD40 mAb induces splenic DC activation in *P. berghei* XAT-infected mice. TCR-δ KO and C57BL/6 WT mice were infected with *P. berghei* XAT by intravenous inoculation of 10^4 iRBCs. The agonistic anti-CD40 mAb (clone 1C10) or control rat IgG was intravenously injected (100 μg per mouse) once on day 4 p.i. (A, C, E, G) Mean fluorescent intensity (MFI) of FITC-conjugated mAbs against MHC-II and co-stimulatory molecules CD40, CD80, and CD86 on DCs ($CD11c^+$ B220- CD3- CD19-) in spleen from each mouse group on day 5 p.i. MFI levels indicate the expression level of each molecule in DCs. (B, D, F, H) Flow cytometry histograms are shown. In each left histogram, the black line indicates the splenic DCs of naïve WT mice, and the grey line indicates the splenic DCs of naïve TCR-δ KO mice. In each middle histogram, the black line indicates the splenic DCs on day 5 p.i. in WT mice administered control rat IgG, the grey line indicates splenic DCs on day 5 p.i. in TCR-δ KO mice administered control rat IgG, and the grey area indicates splenic DCs of naïve WT mice. In each right histogram, the black line indicates the splenic DCs on day 5 p.i. in WT mice administered anti-CD40 mAb, the grey line indicates the splenic DCs on day 5 p.i. in TCR-δ KO mice administered anti-CD40 mAb, and the grey area indicates the splenic DCs of naïve WT mice. (I) Absolute numbers of DCs were measured. $^*P < 0.05$, $^{**}P < 0.01$. $n = 3$ or 4 for each experiment.

anti-CD40 mAb treatment for induction of protective immunity against *Plasmodium* parasites in TCR-δ KO mice remains to be determined. Therefore, we administered the agonistic anti-CD40 mAb to TCR-δ KO mice on days 0, 3, 5, 7, 10, 14 or 17 after infection with *P. berghei* XAT parasites and examined parasitemia (Fig. 3) and survival rate (Table 1). While administration of the anti-CD40 mAb to TCR-δ KO mice at the same time as infection decreased parasitemia around the first peak (from day 5 to 7 p.i.), the TCR-δ KO mice could not control the *Plasmodium* parasites, and all animals eventually died (Fig. 3C and L). In contrast, administration of the anti-CD40 mAb to TCR-δ KO mice on days 3, 5, 7, or 10 p.i. eliminated iRBCs (Fig. 3D–G, M–O). However, elimination of

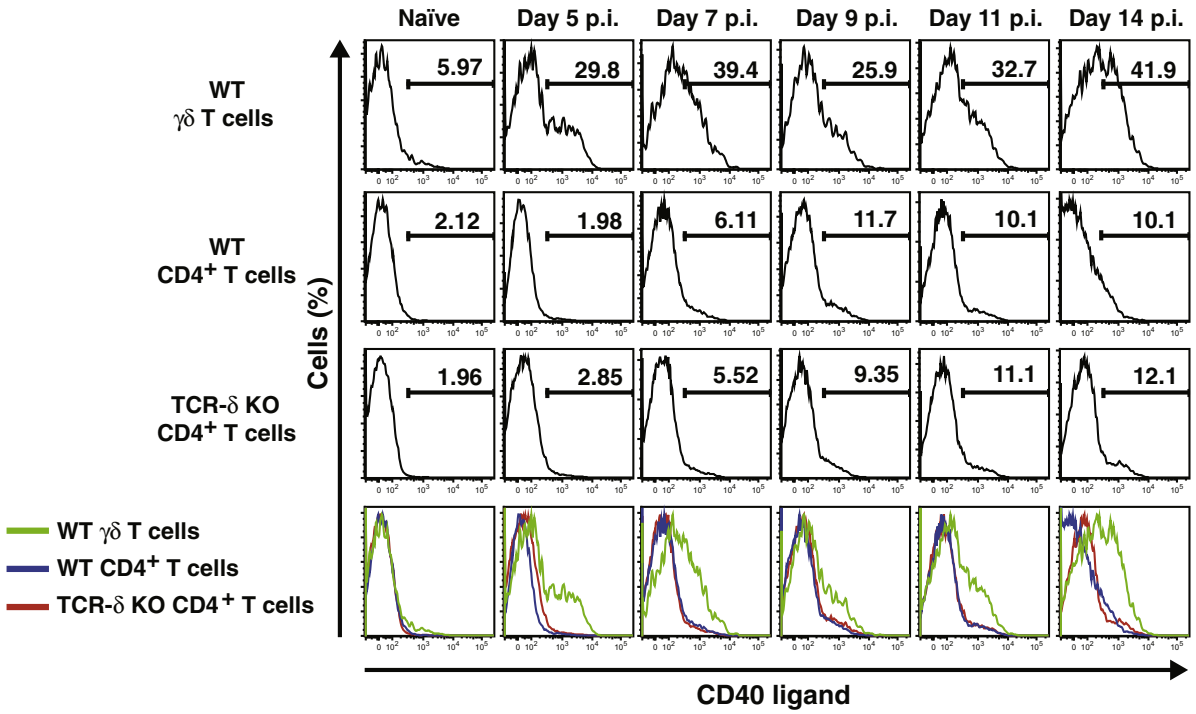

Fig. 2. CD40L expression on γδ T cells and CD4$^+$ T cells in *P. berghei* XAT-infected WT mice. C57BL/6 WT mice were infected with *P. berghei* XAT by intravenous inoculation of 10^4 iRBCs. CD40L-expressing γδ T cells and CD4$^+$ T cells in spleen were measured using flow cytometry. Splenic cells were isolated from naïve mice or *P. berghei* XAT-infected mice on days 5, 7, 9, 11 and 14 p.i. The numbers in the histograms represent the proportion of CD40L$^+$ γδ T cells in spleen from WT mice and of CD4$^+$ T cells in spleen from WT and TCR-δ KO mice.

Plasmodium parasites in mice administered the anti-CD40 mAb was not as great as that in WT mice. Furthermore, administration of the anti-CD40 mAb to TCR-δ KO mice on day 14 or 17 p.i. did not control *Plasmodium* parasites, and all of the TCR-δ KO mice died eventually (Fig. 3H and I). To further examine whether stimulation of CD40 signalling enhanced protective immunity against *Plasmodium* infection, we next analysed WT mice treated with the anti-CD40 mAb. Thus, we administered the agonistic anti-CD40 mAb to WT mice on days 5, 7, 10 or 14 after infection with *P. berghei* XAT parasites and examined parasitemia (Fig. 4). Similar to WT mice that were not administered the anti-CD40 mAb, anti-CD40 mAb-administered WT mice also displayed controlled *P. berghei* XAT parasites. Administration of the anti-CD40 mAb to WT mice on day 5, 7, or 10 p.i. slightly accelerated the last day of parasite elimination (Fig. 4B–D). In contrast, administration of the anti-CD40 mAb to WT mice on day 14 p.i. did not change the time points of parasite elimination (Fig. 4E). These results suggest that stimulation of CD40 signalling in DCs can enhance protective immunity against *P. berghei* XAT parasites during the early phases of infection.

4. Discussion

In this study, we confirmed that splenic DC activation in *P. berghei* XAT-infected mice is enhanced by administration of the agonistic anti-CD40 mAb (Fig. 1). Enhanced DC activation with anti-CD40 mAb administration after *P. berghei* XAT infection led to control of the parasites in TCR-δ KO mice [13]. To elucidate when CD40L-expressing γδ T cells are required for host survival and *P. berghei* XAT elimination, we infected TCR-δ KO mice with parasites and administered the anti-CD40 mAb at various time points after infection. The results showed that protective immunity via CD40–CD40L signalling is important during the early phases of infection (days 3–10 p.i.; Fig. 3).

Administration of the anti-CD40 mAb to TCR-δ KO mice on day 0 p.i. did not provide protective immunity against *P berghei* XAT parasites (Fig. 3). Because DC stimulation stops antigen uptake by phagocytosis, anti-CD40 mAb administration on day 0 p.i. is likely too early for DC stimulation to enhance an antigen-specific immune response [31]. There are several possibilities to explain why administration of the anti-CD40 mAb to TCR-δ KO mice on day 14 or 17 p.i. did not provide protective immunity against parasites. First, DCs may lose their ability to respond to stimulation of CD40 signalling after day 14 or 17 p.i. Second, stimulation of CD40 signalling may activate DCs but may not result in parasite-specific helper T cell responses to eliminate the parasites. The number of CD4$^+$ T cells significantly increased in the spleen of TCR-δ KO mice compared with WT mice. This result suggests that splenic CD4$^+$ T cells from both WT and TCR-δ KO mice receive some stimulation, such as that from IL-2, and expand on day 14 p.i. However, the number of antigen-specific CD4$^+$ T cells in TCR-δ KO mice may be lower than that in WT mice. After infection with *P. berghei* XAT, the proportion of IFN-γ-producing CD4$^+$ T cells and γδ T cells transiently increased in the early phase of infection, but then decreased on days 11 and 14 p.i. [13]. This decrease might be due to inhibitory signalling via PD-1 and CTLA-4, which are inhibitory receptors that suppress TCR signalling, in order to avoid excessive inflammation by T cell activation [32]. In contrast, CD40L expression on CD4$^+$ T cells and γδ T cells was not significantly reduced (Fig. 2). It has been suggested that CD40L-expressing CD4$^+$ T cells could contribute to DC activation [33]. Thus, even in TCR-δ KO mice, DC activation occurred after infection with *P. berghei* XAT [13] (Fig. 1). However, in WT mice, CD40L-expressing γδ T cells could cooperate with CD40L-expressing CD4$^+$ T cells for DC activation, resulting in a higher state of activation.

In our previous report, immunodepletion of γδ T cells at day 9 p.i. controlled *P. berghei* XAT. Therefore, we considered that γδ T cells play important roles in protective immunity before day 9

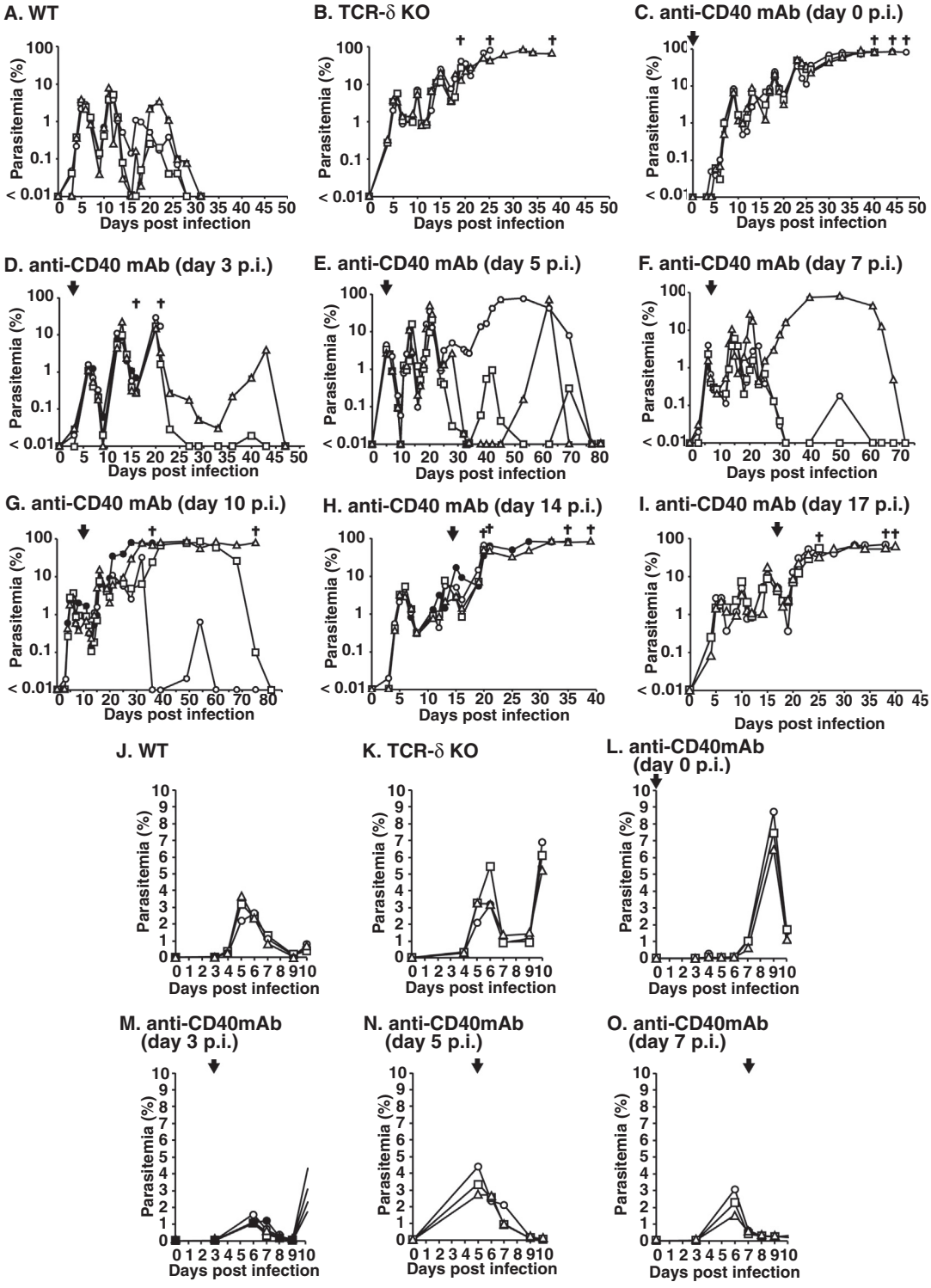

Fig. 3. The effect of agonistic anti-CD40 mAb treatment on the control of *P. berghei* XAT parasites in TCR-δ KO mice. (A–O) Time course of parasitemia. WT (A, J) and TCR-δ KO mice (B–I, K–O) were infected with *P. berghei* XAT through intravenous inoculation of blood-stage parasites (10^4 iRBCs), and parasitemia was measured. *P. berghei* XAT-infected TCR-δ KO mice were intravenously administered the agonistic anti-CD40 mAb on days 0 (C, L), 3 (D, M), 5 (E, N), 7 (F, O), 10 (G), 14 (H), or 17 (I) p.i. Parasitemia was shown from day 0 to 10 p.i. and was graphed on a linear scale (J, M, N, O). $n = 3–5$ for each experiment. Arrows indicate the time points of mAb treatment. Daggers indicate death.

p.i. at least. [13]. However, the anti-CD40 mAb treatment was partially effective for providing protective immunity to *Plasmodium* in TCR-δ KO mice even on day 10 p.i. These results may be due to stronger stimulation of CD40 signalling by anti-CD40 mAb treatment than by γδ T cells. In this study, we used not only TCR-δ KO mice but also WT mice to investigate the time at which CD40–CD40L signalling is important for protective immunity against *Plasmodium* parasites. The results of those kinetic analyses of *P. berghei* XAT infection in WT mice administered the anti-CD40 antibody support our hypothesis that stimulation of CD40

Table 1
Survival rate of mice treated with anti-CD40 mAb at different times during *P. berghei* XAT infection.

Mouse	Time of anti-CD40 mAb administration (Days post-infection)	Survival rate
WT	–	6/6
TCR-δ KO	–	0/6
	0	0/7
	3	3/7
	5	7/7
	7	7/7
	10	3/7
	14	0/7
	17	0/3

signalling in DCs enhances protective immunity against *P. berghei* XAT parasites during the early phases of infection. However, in contrast to TCR-δ KO mice, WT mice possess CD40L-expressing γδ T cells and can control *P. berghei* XAT parasites *without* administration of the anti-CD40 antibody. In WT mice, CD40L–CD40 signalling should sufficiently activate DCs and induce protective immunity against *P. berghei* XAT parasites. We could not exclude the possibility that, in the case of WT mice, administration of the anti-CD40 antibody caused activation of phagocytic activity, resulting in accelerated parasite elimination. Thus, further detailed analyses using WT mice treated with the anti-CD40 mAb are important for determining whether stimulation of CD40 signalling enhances protective immunity against *Plasmodium* infection.

During the first peak of parasitemia, phagocytic cells, such as dendritic cells and macrophages, play a role in the innate response. $CD4^+$ T cells began to activate before the start of the second peak of parasitemia [13] (from day 7 p.i.). Thus, during the second and third parasitemia peaks, *P. berghei* XAT antigen-specific $CD4^+$ T cells would respond to *P. berghei* XAT parasites and contribute to parasite regulation. Furthermore, a previous report showed that antibody-dependent phagocytosis was required for the control of *P. berghei* XAT parasites. That report suggests that B cells activate and differentiate into plasma cells, for the production of *P. berghei* XAT antigen-specific antibodies. Such antibodies would be important for the elimination of the *P. berghei* XAT parasites. As shown in our previous report on *P. berghei* XAT-infected WT mice [25], the level of antigen-specific antibodies in plasma increased markedly during the third peak of parasitemia. Thus, during that third peak, B cells and plasma cells may respond to *P. berghei* XAT parasites – an acquired immune response – resulting in the elimination of the parasites. As described above, our previous report showed that the ability of WT mice to control *P. berghei* XAT parasites was not influenced by γδ T cell depletion from day 10 p.i. [13]. Thus, the CD40L-expressing γδ T cells seem to play an indirect role in the immune response against the *P. berghei* XAT parasites during the second and third peaks of parasitemia.

A previous report regarding cancer immunity showed that treatment of mice with a combination of anti-CD40 mAb and IL-2 improves survival after primary tumour injection, but decreases rejection against secondary tumour injection after immunisation with irradiated tumour cells [34]. The impaired secondary antitumor responses were caused by the loss of memory T cells. It is

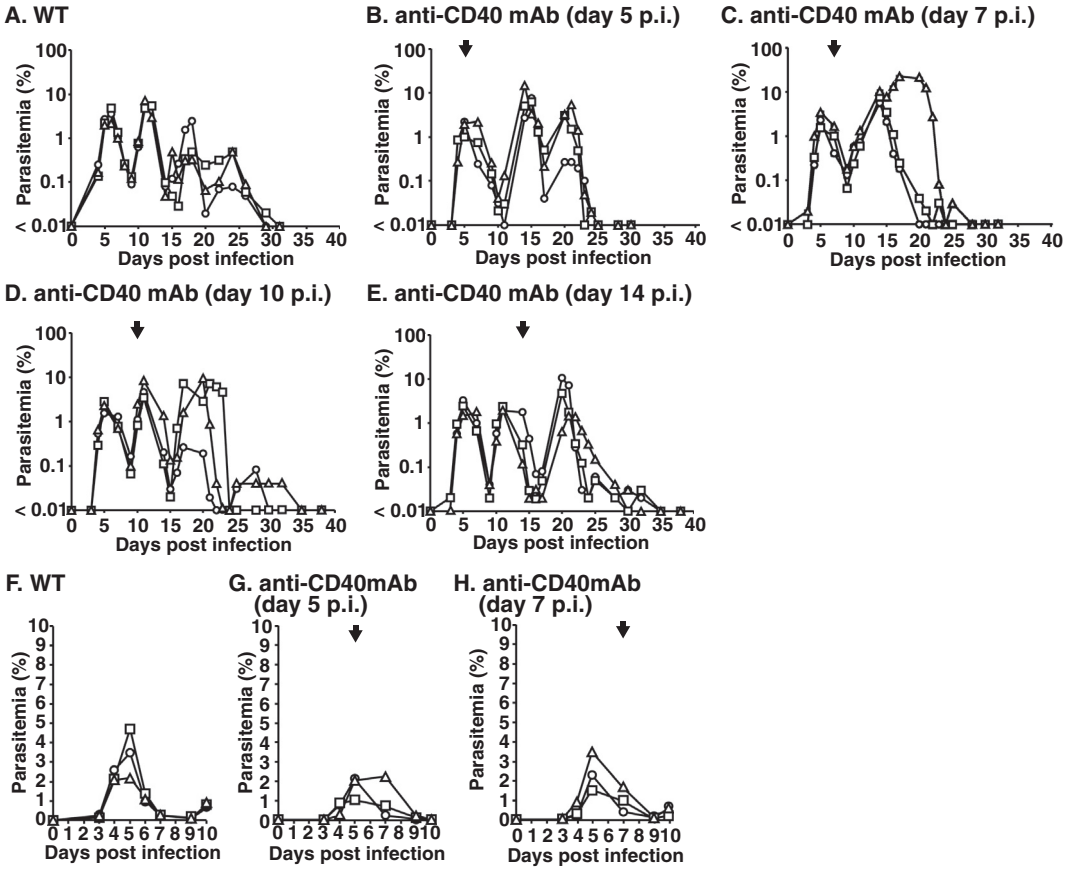

Fig. 4. Effects of agonistic anti-CD40 mAb treatment on the control of *P. berghei* XAT parasites in WT mice. (A–H) Time course of parasitemia. WT mice were infected with *P. berghei* XAT through intravenous inoculation of blood-stage parasites (10^4 iRBCs), and parasitemia was measured (A, F). *P. berghei* XAT-infected WT mice were intravenously administered the agonistic anti-CD40 mAb on day 5 (B, G), 7 (C, H), 10 (D), or 14 (E) p.i. Parasitemia was shown from day 0 to 10 p.i. and was graphed on a linear scale (F, G, H). $n = 3$ for each experiment. Arrows indicate the time points of mAb treatment.

important to investigate whether impaired memory function also occurs in malaria after stimulation of CD40 signalling. Studies regarding the role of CD40 signalling in association with long-term T cell responses should advance the understanding of protective immunity against malaria.

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