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SHORT COMMUNICATION

$CD4^+$ T cell response in early erythrocytic stage malaria: *Plasmodium berghei* infection in BALB/c and C57BL/6 mice

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Abstract *Plasmodium berghei* ANKA causes lethal malaria in mice. It is well established that C57BL/6 mice die early with fulminant symptoms including convulsion, whereas BALB/c mice survive this phase and die later of anemia and prostration. Early death in C57BL/6 mice has been considered to result from the adverse effects of inflammatory cytokines. To elucidate the $CD4^+$ T cell responses in early death due to severe malaria, the kinetics of $CD4^+$ T cells were compared by analyzing cell surface markers and the production of cytokines and transcription factors. The results revealed that cytokine production by $CD4^+$ T cells was induced as early as 5 days after infection and the maintenance of higher levels of IL-4 and IL-10 may be associated with the protection of BALB/c mice from early death. These results suggest that parasite control in the early phase of infection may be important for the development of an effective vaccine.

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Malaria claims more than one million lives every year worldwide (Snow et al. 2005; WHO 1992). Among four species of *Plasmodium* parasites that cause human malaria, *Plasmodium falciparum* is by far the most malignant species and causes severe malaria, inducing cerebral symptoms and anemia that often result in death. Previous studies have revealed the complex pathogenesis of malaria, including (1) the adhesion of parasite-infected erythrocytes to endothelial cells that results in microthrombi in various organs, causing multiple organ failure (Mackintosh et al. 2004) and (2) the production of various inflammatory cytokines such as IFN-γ, which is produced by Th1, $CD8^+$ T, γδT, and NK cells, and TNF-α and IL-6 that are produced by NK cells and monocytes/macrophages (Armah et al. 2007; Nie et al. 2007; Langhorne et al. 2004).

The murine malaria parasite *Plasmodium berghei* ANKA causes similar severe disease in mice and has been used as a model of human *P. falciparum* infection (Yanez et al. 1996; de Kossodo and Grau 1993; Griffith et al. 2007). It is well established that C57BL/6 mice, which show a Th1-biased phenotype, die of severe malaria in the early phase of infection, while BALB/c mice that show a Th2-biased phenotype survive this period and then eventually die of anemia and prostration infection (Yanez et al. 1996; de Kossodo and Grau 1993; Griffith et al. 2007). Because the symptoms in C57BL/6 mice mimic human severe malaria, the immunological responses involving $CD4^+$ T cells were compared in these two strains.

Although excessive production of inflammatory cytokines may lead to early death by cerebral malaria (Nie et al. 2007; Yanez et al. 1996; de Kossodo and Grau 1993; Haque et al. 2001), these cytokines could also protect hosts by promoting the exclusion of parasites in early infection (Perlmann et al. 1998). IFN-γ, a Th1 cytokine, activates

macrophages to produce IL-12, oxygen radicals, and carbon monoxide, which have parasiticidal activity. TNF-α activates NF-κB and MAPK to induce the production of inflammatory cytokines including IL-6, which promotes antibody production, B cell proliferation, and T cell differentiation and activation. Antibodies of IgG1 and IgG3 subclasses opsonize macrophages to phagocytoze parasite-infected erythrocytes. On the other hand, regulatory cytokines such as IL-10 and TGF-β may prohibit early death through the suppression of Th1 responses (Nie et al. 2007; Riley et al. 2006). IL-4 produced by Th2 cells is known to promote the exclusion of parasites in late infection (Perlmann et al. 1998).

$CD4^+$ T cells produce various cytokines and play pivotal roles in immune responses (Cruz Cubas et al. 1994). $CD4^+$ T cells are now classified into four subsets: Th1, Th2, Th17, and Treg cells according to the cytokines they produce and the functions they perform (Weaver et al. 2007). Generally, Th1 cells help to protect hosts from bacteria or virus infection, which requires mainly cellular immunity, while Th2 cells help to protect against helminth infection that requires mainly humoral immunity. Th17 cells, which have been characterized recently, play important roles in inflammatory and autoimmune responses (Weaver et al. 2007). At present, there are no reports concerning Th17 responses against malaria parasite infection. $CD4^+CD25^+$ regulatory T (Treg) cells (Sakaguchi et al. 1995) suppress host immune responses and promote the proliferation of pathogens leading to persistent infection (Belkaid and Rouse 2005). There are contradictory reports on the roles of Treg cells in malaria, depending on the host and parasite species. Several researchers reported that Treg cells suppress the host immune system and aggravate malaria both in human and mice (Belkaid and Rouse 2005; Walther et al. 2005; Hisaeda et al. 2004; Long et al. 2003). On the other hand, it was also shown that Treg cells protect hosts through suppression of severe inflammation (Seixas and Ostler 2005).

When a mosquito vector bites a vertebrate host, sporozoite stage malaria parasites first proliferate in the liver and then infect red blood cells. It is generally believed that the liver stage parasites do not induce immunological reactions, though some researchers think otherwise (Doolan and Martinez-Alier 2006; Hoffman et al. 1990). Immune responses against sporozoites are mediated by $CD8^+$ T cells, which attack parasitized liver cells. $CD4^+$ T cells help $CD8^+$ T cells through the production of IFN-γ and IL-4.

Although the blood stage parasites play major roles in the pathogenesis of malaria, the lack of MHC on erythrocytes hampers cellular immunity, leaving antibodies as the only functional modality to remove parasites. However, parasites can use antigen variation or mutation to circumvent protection by antibodies. On the other hand, it was shown that cytokine-producing $CD4^+$ T cells are essential to protective immunity (Stephens and Langhorne 2006; Cockburn and Zavala 2007; Langhorne 1994).

Thus, $CD4^+$ T cells a play pivotal role in protection against malaria in innate immunity, antibody production, and cellular immunity by producing various cytokines. However, $CD4^+$ T cell responses in early erythrocytic stage parasites have not been well studied because it is difficult to analyze the weak immune responses against small numbers of parasites. Recently, real-time quantitative polymerase chain reaction (PCR) has enabled the analysis the very low levels of gene expression. In this report, the various cytokines produced by $CD4^+$ T cells were measured every 2 up to 11 days post-infection (DPI) after erythrocytic stage malaria infection.

First, the survival curve after *P. berghei* infection in C57BL/6 and BALB/c mice was confirmed. As shown previously, infected C57BL/6 mice all died early, from 7 to 9 DPI, showing convulsions, shivering, and piloerection, while most BALB/c mice survived this period without any symptoms (Fig. 1a). Then parasitemia was compared between C57BL/6 and BALB/c mice. Parasitemia was higher in C57BL/6 mice than in BALB/c mice from 2 to 9 DPI and significantly ($p<0.05$) higher from 3 to 9 DPI, which was confirmed in three independent experiments (Fig. 1b). The body weights of the mice were similar in both strains (data not shown). Infection efficiency of inoculated parasites and doubling time were estimated by linear extrapolation from the parasitemia between 2 and 4 DPI. The infection efficiency was 60% for C57BL/6 mice and 80% for BALB/c mice, and the doubling times were 6.0 h and 7.2 h, respectively. To our knowledge, this is the first report of such a difference in the increase of parasitemia between C57BL/6 and BALB/c mice. It was reported that Th1 cytokines suppress the proliferation of malaria parasites in vitro (Cruz Cubas et al. 1994; Ferrante et al. 1990). However, the estimated doubling time indicated that proliferation is accelerated in Th1-biased C57BL/6 mice. Parasitemia is a function of parasite growth, elimination by immune response, and possibly redistribution in the body. Further studies focused on early immune responses may provide further information about this phenomenon.

Next, the numbers of splenic $CD4^+$ and $CD4^+CD25^+$ T cells were compared between C57BL/6 and BALB/c mice in the early infection phase. The total number of the splenic $CD4^+$ T cells started to increase at 5 DPI in both strains and remained generally higher in BALB/c mice but decreased to the original level at 7 DPI in CL57B/6 mice. The peak level was lower in the CL57B/6 mice (Fig. 2a). The total number of the splenic $CD4^+CD25^+$ T cells showed a similar profile to total $CD4^+$ T cells in both strains (Fig. 2b). The differences between the two strains were statistically significant (ANOVA, $p<0.05$). The percentage of $CD25^+$

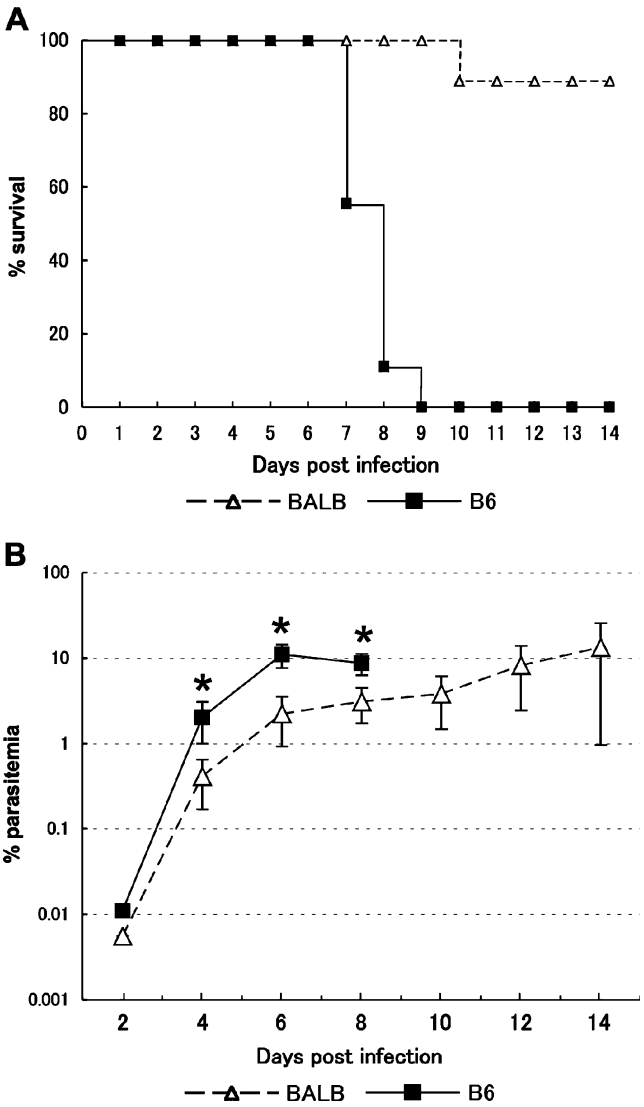

Fig. 1 Comparison of survival and parasitemia between 6-week-old female BALB/c (BALB) and C57BL/6 (B6) mice after intraperitoneal *P. berghei* ANKA infection (1×10^6 infected erythrocytes/mouse, $n=9$). **a** The percentage survival was monitored daily. **b** Parasitemia was counted from Giemsa-stained blood smear preparations. Each point represents the mean parasitemia±SD of the surviving animals. *$p<0.05$ by Student's *t*-test

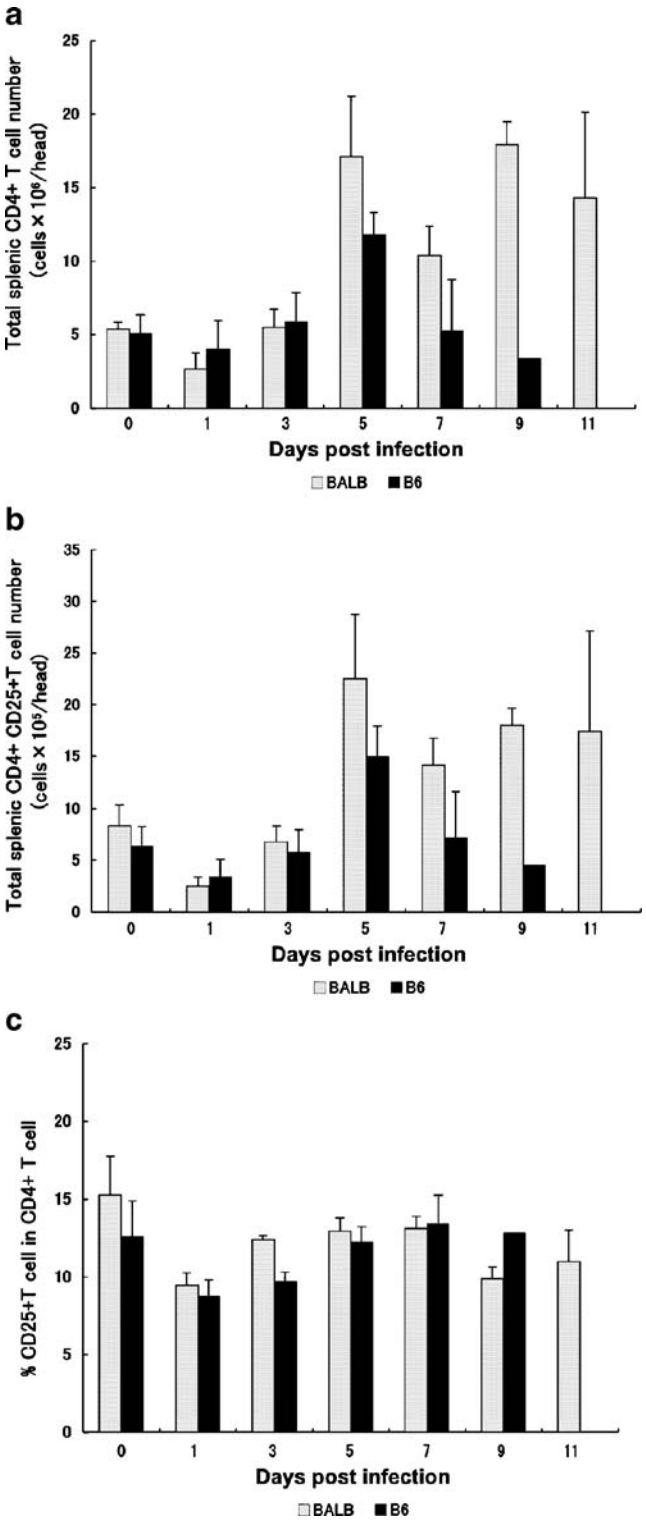

Fig. 2 Kinetics of splenic $CD4^+$ T cells (**a**), $CD4^+$ $CD25^+$ T cells (**b**), and percentage of $CD25^+$ T cells in splenic $CD4^+$ T cells (**c**) during *P. berghei* ANKA infection. Total $CD4^+$ T cells were isolated from infected BALB/c (BALB) and C57BL/6 (B6) mice spleen using a BD™ IMag Anti-Mouse CD4 Particles-DM (GK1.5) kit (BD Pharmingen, San Diego, CA, USA). The purity of $CD4^+$ T cell fractions estimated by flow cytometric analysis was 93–99%. Flow cytometric analyses were performed using PE-conjugated anti-mouse CD4 (RM4-5) and FITC-conjugated anti-mouse CD25 mAb (PC61). The absolute numbers of T cells (**a**, **b**) were calculated. The differences between BALB and B6 mice in **a** and **b** were statistically significant (ANOVA, $p<0.05$). At least three mice were used to measure each time point. Data are representative of four independent experiments with similar results

cells in splenic $CD4^+$ T cells was constant, i.e., about 10~15%, after inoculation in both strains (Fig. 2c). Malaria is a systemic disease and antigen presentation and lymphocyte activation occur mostly in the spleen. Thus, the lower T cell proliferation rate may reflect the weaker immunological

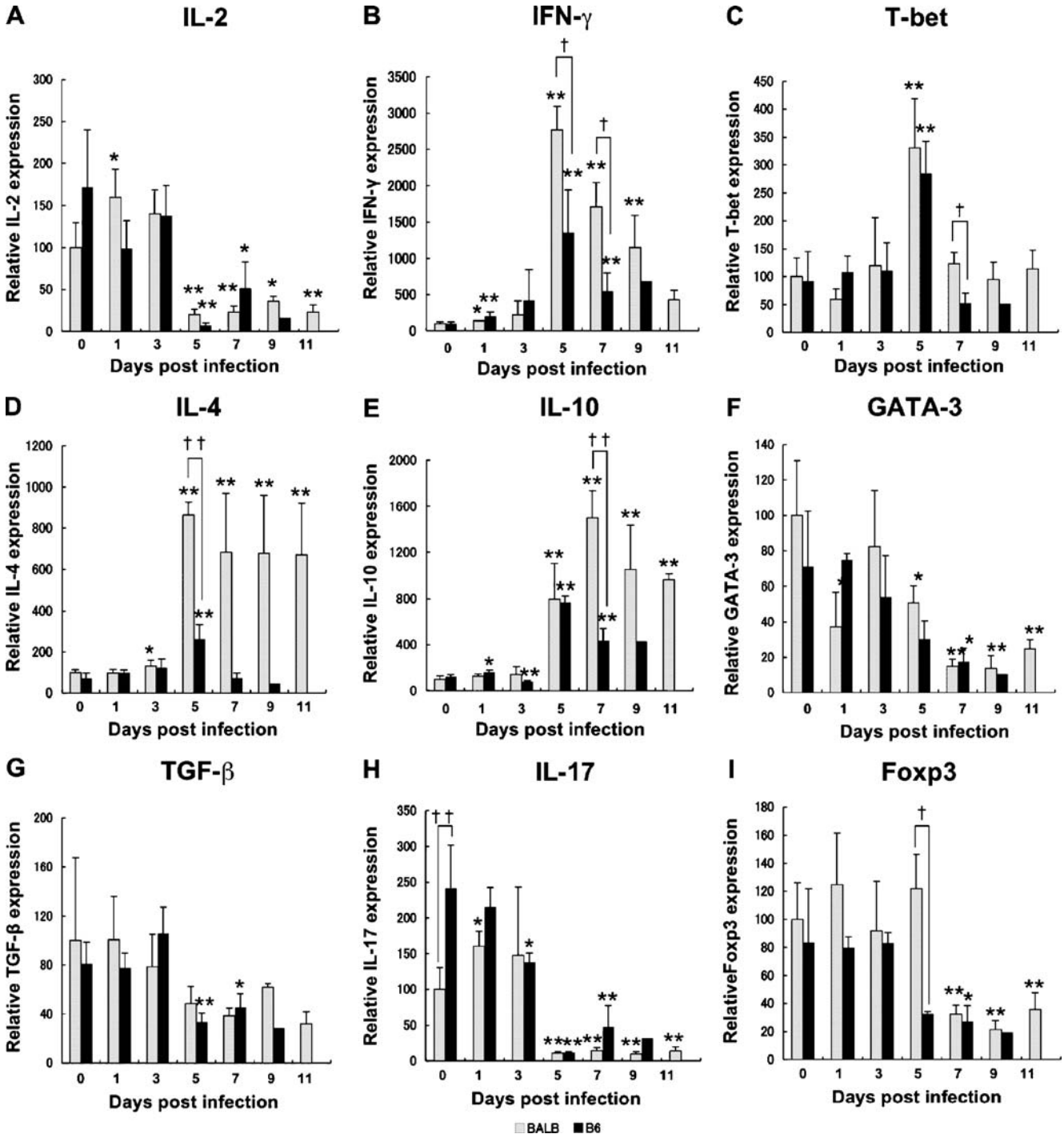

Fig. 3 Kinetics of cytokine- and transcription factor-genes expressed by splenic $CD4^+$ T cells during *P. berghei* ANKA infection. Total RNA was isolated from $CD4^+$ T cells and mRNA expression levels of IL-2 (**a**), IFN-γ (**b**), T-bet (**c**), IL-4 (**d**), IL-10 (**e**), GATA-3 (**f**), TGF-β (**g**), IL-17 (**h**), and Foxp3 (**i**) were quantified using real-time RT-PCR. Primers optimized for real-time PCR were purchased by TaKaRa (Osaka, Japan). Data were normalized to HPRT-1 gene expression in individual samples and average gene expression levels in BALB/c mice at 0 DPI were defined as 100. Each experiment is representative of three separate infections. Each *bar* represents the mean of three samples±SD. BALB: BALB/c mice (*gray bars*), B6: C57BL/6 mice (*black bars*). *$p<0.05$; **$p<0.01$ (between day0 and the indicated DPI); *single dagger* $p<0.05$; *double dagger* $p<0.01$ (between BALB and B6) by Student's *t*-test

responses in C57BL/6 mice, suggesting a relationship with early death.

To compare the dynamics of $CD4^+$ T cell subsets in the early phase of infection, the expression levels of various cytokine genes and transcription factors in splenic $CD4^+$ T cells were periodically measured during the course of early infection using real-time quantitative RT-PCR (Fig. 3). IL-2 gene expression decreased abruptly in both strains at 5 DPI and remained suppressed (Fig. 3a). It is reported that IL-2 production was reduced within 7 days after infection, resulting in decreased T cell proliferation (Nie et al. 2007). IFN-γ, a Th1 cytokine, showed an abrupt increase at 5 DPI and then decreased gradually. Contrary to expectations, the IFN-γ level was higher in Th2-biased BALB/c mice than in Th1-biased CL57B/6 mice (Fig. 3b). The expression of T-bet, a transcription factor that promotes the differentiation of Th1 cells, showed a transient increase at 5 DPI (Fig. 3c) in both strains. At 7 DPI, T-bet expression decreased quickly to the original level in BALB/c mice but only to 50% in C57BL/6 mice. This difference was statistically significant and reduced T-bet expression in C57BL/6 mice may be related to the different clinical outcomes.

The expression levels of IL-4 and IL-10 genes, which are both Th2 cytokines, showed abrupt increases at 5 DPI (Fig. 3d, e). This increase was more marked and persistent in BALB/c mice but was transient in C57BL/6 mice. Because IL-4 induces Th2 differentiation, B cell proliferation, and class switching of antibodies while suppressing Th1 differentiation, elevated IL-4 in BALB/c mice with subsequent antibody production may be functional in protection from early death. IL-10 promotes B cell proliferation and antibody production. It was shown that increased levels of $CD4^+$ T cells, which produce IL-10 in primary infection, play a role in anti-disease immunity using MSP-1 TCR transgenic mice (Stephens et al. 2008). IL-4 and IL-10 may protect BALB/c mice from early death by B cell activation. Gene expression levels of the Th2 cytokine IL-6 showed neither an increase nor decrease in C57BL/6 mice but decreased at 5 DPI in BALB/c mice (data not shown). The expression level of GATA-3, a transcription factor that promotes Th2 differentiation, started to decrease at 5 DPI in both strains (Fig. 3f).

The expression level of Foxp3, the master transcription factor of Treg cells, decreased at 5 DPI in CL57B/6 mice but decreased at 7 DPI in BALB/c mice (Fig. 3i). However, TGF-β, which is produced by Treg cells, decreased at 5 DPI and remained low in both mice strain (Fig. 3g). This paradox should be elucidated in further studies. Total $CD4^+CD25^+$ T cell numbers remained elevated in BALB/c mice even after Foxp3 levels decreased at 7 DPI, suggesting that a fraction of the increased $CD4^+CD25^+$ T cells represented activated T cells rather than Treg cells (Fig. 2b). The present findings showed that C57BL/6 mice started to die at 7 DPI (Fig. 1a), when Foxp3 expression decreased (Fig. 3i), suggesting that Treg cells may have no correlation with early death in the system of *P. berghei* ANKA infection in C57BL/6 mice.

The levels of IL-17 gene expression, which is produced by Th17 cells, decreased remarkably in both strains at 5 DPI and remained low thereafter, suggesting that these cells are not involved in either protection or early death (Fig. 3h), or the reduction in IL-17 level may be related to the progress of infection.

Overall, the levels of cytokine production in C57BL/c mice were lower than that those of BALB/c mice (Fig. 3), suggesting that early death in C57BL/6 mice is caused by lower immune response rather than cytokine storm. Furthermore, it is suggested that elevated IL-4 and IL-10 levels in BALB/c may protect from early death by promoting antibody production (Fig. 3d, e).

In conclusion, the immune responses elucidated in this study indicated that 5 DPI is the turning point in both mouse strains, while early death in C57BL/6 mice started from 7 DPI in this model. To date, studies on vaccine development using mouse models have usually focused on the late infection phase, but the present study showed that the early phase is very important. The development of a malaria vaccine to control parasitemia or $CD4^+$ T cell responses up to 5 DPI should be pursued using this infection model to protect hosts from early death.

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