■ ORIGINAL ARTICLE

Age-Related CD4+CD25+Foxp3+ Regulatory T-Cell Responses During *Plasmodium berghei* ANKA Infection in Mice Susceptible or Resistant to Cerebral Malaria

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Abstract: Different functions have been attributed to CD4+CD25+Foxp3+ regulatory T-cells (Tregs) during malaria infection. Herein, we describe the disparity in Treg response and pro- and anti-inflammatory cytokines during infection with *Plasmodium berghei* ANKA between young (3-week-old) and middle-aged (8-month-old) C57BL/6 mice. Young mice were susceptible to cerebral malaria (CM), while the middle-aged mice were resistant to CM and succumbed to hyperparasitemia and severe anemia. The levels of pro-inflammatory cytokines, such as TNF-α, in young CM-susceptible mice were markedly higher than in middle-aged CM-resistant mice. An increased absolute number of Tregs 3-5 days post-inoculation, co-occurring with elevated IL-10 levels, was observed in middle-aged CM-resistant mice but not in young CM-susceptible mice. Our findings suggest that Treg proliferation might be associated with the suppression of excessive pro-inflammatory Th1 response during early malaria infection, leading to resistance to CM in the middle-aged mice, possibly in an IL-10-dependent manner.

Key words: *Plasmodium berghei* ANKA, cerebral malaria, CD4+CD25+Foxp3+ regulatory T cell, age, cytokine

INTRODUCTION

Cerebral malaria (CM) is one of the most severe complications of *Plasmodium* infection and a major cause of death, primarily afflicting children aged 2-6 years in sub-Saharan Africa [1]. CM appears to be mediated more by immunopathological host responses to infection than by the parasite per se [2,3]. Studies on malaria have progressively shown an important role for overwhelming the pro-inflammatory Th1 pathway in CM pathogenesis [4,5] and the subsequent combined effects of sequestration of parasitized red blood cells within blood vessels in the brain. However, the precise mechanism responsible for neuropathology remains unknown. Excessive serum levels of pro-inflammatory cytokines have been implicated in

the pathogenesis of CM in murine models and human studies, with an association between higher mortality rate and elevated pro-inflammatory cytokine levels [6,7]. However, several have also revealed that pro-inflammatory cytokines are critical for the successful control and resolution of malaria infection in both humans and murine models [8]. A weak pro-inflammatory response may lead to persistence and replication of parasites, while an excessive pro-inflammatory response may result in immunopathological consequences such as CM. Therefore, induction of an appropriate and effective immune response to malaria infection is needed for the host to subsequently control and eliminate this pathogen.

CD4+CD25+Foxp3+ regulatory T cells (Tregs) play determinant roles in the preservation of self-tolerance and in the control of graft and tumor rejection and inflammation. Their abrogation leads to autoimmunity and inflammatory diseases in several experimental models [9,10]. Tregs also participate in the control of overwhelming responses to infectious agents such as viruses, bacteria, and protozoan parasites [11,12]. In malaria, Tregs expand during infection with the *Plasmodium*

berghei ANKA strain [13,14] and have been shown to inhibit the development of pathogenic Th1 cells, responsible for cerebral disease in resistant BALB/c mice [13]. These results contrast with the detrimental effect associated with Tregs during *P. berghei* ANKA infection in susceptible C57BL/6 mice [14,15]. In this infection model, depletion of Tregs results in a significant increase in survival, a minor but significant reduction in blood parasitemia, and an important reduction in parasite load in the brain and vasculature. A comparable delay in the onset of peak parasitemia has been reported during *P. berghei* NK65 infection in mice depleted of Tregs [16], and in the *P. yoelii* 17XL infection model the elimination of Tregs allows BALB/c mice to control otherwise lethal infections [17]. Moreover, during *Plasmodium falciparum* infection in humans, the expansion of natural Tregs and the production of transforming growth factor-β (TGF-β) is correlated with higher parasite multiplication rates [18,19]. Altogether, these observations attribute contrasting functions to natural Tregs during *Plasmodium* infections.

A study on age-related susceptibility and resistance to *P. berghei* in mice revealed that 70% of 4-week-old C57BL/6 mice died from CM. However, only 20-30% of 10- and 16-week-old C57BL/6 mice developed CM [20]. Our previous studies have shown that activation of Tregs is correlated with susceptibility in *P. yoelii* 17XL-infected mice. Tregs can regulate the Th1 response by modifying dendritic cells whose expansion as well as increased IL-12 production following infection provide important co-stimulatory and cytokine signals to support the proliferation and activation of Th1 cells [21]. Tregs mediate the incidence and outcome of CM in *P. berghei* ANKA-infected mice by modifying the pro-inflammatory responses [22-24]. Herein, we compared the infection course and Treg response in young (3-week-old) and middle-aged (8-month-old) C57BL/6 mice infected with *P. berghei* ANKA in order to elucidate the importance of Tregs in CM.

MATERIALS AND METHODS

Mice, parasites, and experimental infection

Female 3-week-old (young) and 8-month-old (middle-aged) C57BL/6 mice were purchased from Beijing Animal Institute (Beijing, China). *P. berghei* ANKA was kindly provided by Dr. Motomi Torii (Department of Molecular Parasitology, Ehime University Graduate School of Medicine, Ehime, Japan). Infections were initiated by intraperitoneal (i.p.) injection of 1×10^6

P. berghei ANKA parasitized erythrocytes for each group of young and middle-aged C57BL/6 mice. Parasitemia was monitored by counting the number of parasite-infected erythrocytes per 1,000 erythrocytes by light microscope examination of Giemsa-stained, thin (tail) blood smears. All experiments were performed in compliance with local Animal Ethics Committee requirements.

Spleen cell culture

Splenocyte culture was performed as previously described [25]. Briefly, spleens from normal and infected mice were removed aseptically and pressed through a sterile fine-wire mesh with 10 ml RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 25 mM HEPES, 0.12% gentamicin, and 2 mM glutamine. Cell suspensions were collected by centrifuging at 350 *g* for 10 min. Erythrocytes were lysed with cold 0.17 M NH_4Cl, and cells were washed twice with fresh medium. The viability of cells was determined by trypan blue exclusion and was always greater than 90%. Aliquots (500 µl/ well) of cell suspensions (1×10^7/ml) were incubated in 24-well flat-bottom tissue culture plates (Falcon) in triplicate for 48 hr at 37℃ in a humidified 5% CO_2 incubator. Supernatant fractions were collected and stored at -80℃ until they were assayed.

Flow cytometry analysis

A portion of the simultaneously infected mice was sacrificed at the indicated time to detect Tregs. Spleen cells from C57BL/6 mice were collected at different time points after infection. To assess Tregs, FITC-anti-CD4 and PE-anti-CD25 antibodies (clone PC61) were added to spleen cells and resuspended in 100 µl of PBS supplemented with 3% FCS for surface staining. The cells were fixed and permeabilized and intracytoplasmic staining was performed using APC-anti-Foxp3 (clone FJK16s) antibody. The cells were washed twice with PBS containing 1% FCS and resuspended in 300 µl of PBS. The cells were analyzed in a FACSCalibur cytofluorometer using CellQuest software. Viable cells were gated by forward and side scattering. Unless otherwise indicated, antibodies were purchased from BD Biosciences (San Jose, California, USA).

Detection of cytokines by ELISA

Levels of IFN-γ, TNF-α, and IL-10 were measured by commercial ELISA kits according to the manufacturer's protocol (R&D Systems, Minneapolis, Minnesota, USA). The OD values

were read in a microplate reader at 450 nm. The concentration of cytokines in each sample was calculated using a standard curve generated using recombinant cytokines.

Statistical analysis

Data are presented as the mean ± standard error of the mean (SE). Statistical significance of the differences was analyzed using the Student t-test or 1-way ANOVA (SPSS 17.0). A value of $P < 0.05$ was considered significant.

RESULTS

Course of infection in young and middle-aged C57BL/6 mice

Young and middle-aged C57BL/6 mice infected with *P. berghei* ANKA showed divergent courses of infection and disease severity. The young mice succumbed to *P. berghei* ANKA-mediated CM on day 6 to 10 post-infection (PI) with characteristic signs of CM, including ruffled hair coat, rapid respiration, and lack of mobility, and parasitemia around 7%. Although the middle-aged mice died at day 13 to day 20 PI, the neurological signs of CM were not exhibited in this group, with a peak parasitemia of approximately 40% (Fig. 1). These results indicated that the middle-aged C57BL/6 mice were resistant to CM during *P. berghei* ANKA infection.

Pro-inflammatory cytokine levels in young and middle-aged mice

CM is hypothesized to result from a strong host pro-inflam-

matory response mediated by cytokines. Here, we determined the concentrations of IFN-γ and TNF-α in the supernatants of splenocyte cultures from young and middle-aged mice by ELISA. Infection led to significantly increased IFN-γ production on day 5 PI in both young and middle-aged mice ($P < 0.01$). The IFN-γ level was higher in middle-aged mice than in young mice, but the difference was not statistically significant (Fig. 2A).

The change in TNF-α production by splenocytes differed between the young and middle-aged mice. TNF-α levels in young mice increased significantly on days 3 ($P < 0.01$) and day 5 PI ($P < 0.05$), whereas there was a significant elevation in middle-aged mice on day 5 PI only ($P < 0.05$). On day 3 PI, the TNF-α level in young mice was significantly higher than in middle-aged mice ($P < 0.05$, Fig. 2B).

Our results showed that infection with malaria-causing parasites induced production of inflammatory cytokines, and the outcome of the infection was associated with the level and time of cytokine production.

Treg, CD4+ T cell, and IL-10 levels in young and middle-aged mice

To compare the immunoregulatory effects of Tregs in young and middle-aged mice during malaria parasite infection, flow cytometric analysis was performed. By triple staining with FITC-anti-CD4, PE-anti-CD25, and APC-anti-Foxp3 monoclonal antibodies, Tregs in splenocytes from young and middle-aged mice infected with *P. berghei* ANKA were evaluated. As shown in Fig. 3A, there was no significant change in the number of Tregs in young mice at each time point compared with unin-
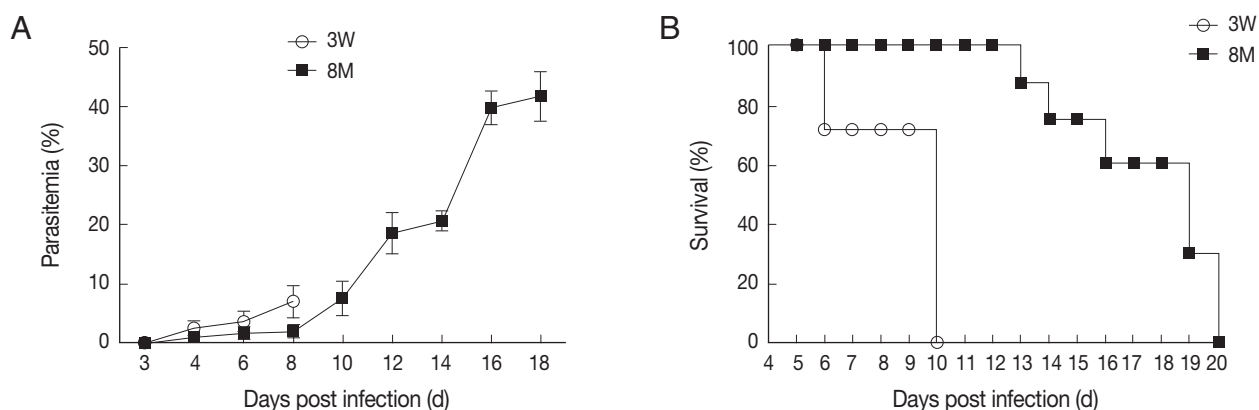

Fig. 1. Parasitemia (A) and survival rate (B) of young and middle-aged mice infected with *P. berghei* ANKA. Percentages of parasitemia were calculated by counting the number of parasite-infected erythrocytes per 1,000 total erythrocytes by light microscope examination of Giemsa-stained, thin (tail) blood smears. Mortality was checked daily. Results are presented as the arithmetic mean of 9 mice per group ± SE.
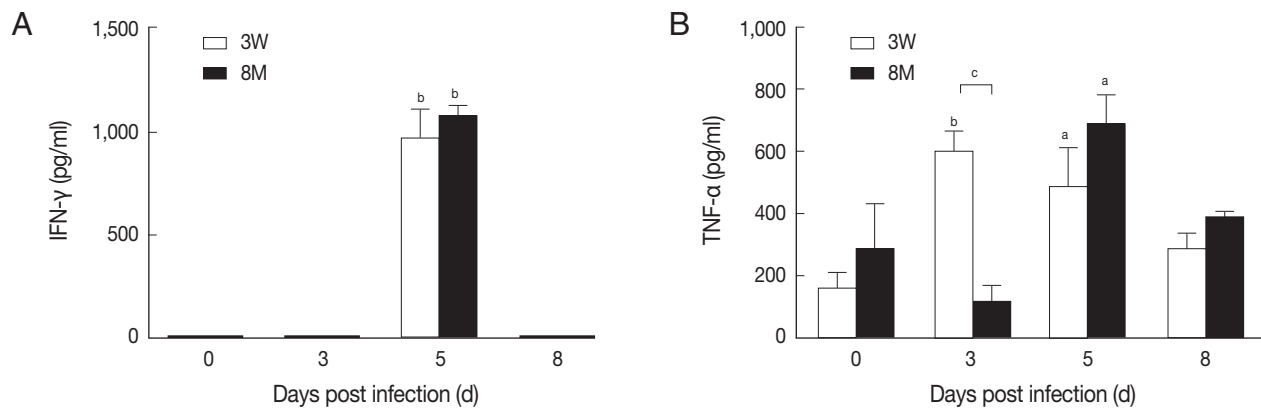
Fig. 2. Level of IFN-γ and TNF-α in young and middle-aged mice with *P. berghei* ANKA infection. ELISA was performed to detect the level of IFN-γ and TNF-α in supernatants of cultured spleen cells from infected mice. Results are presented as the arithmetic mean of 9 mice per group±SE. [a]$P<0.05$, [b]$P<0.01$ versus corresponding values for uninfected control mice (day 0 PI). [c]$P<0.05$ indicates the comparison between young and middle-aged mice.
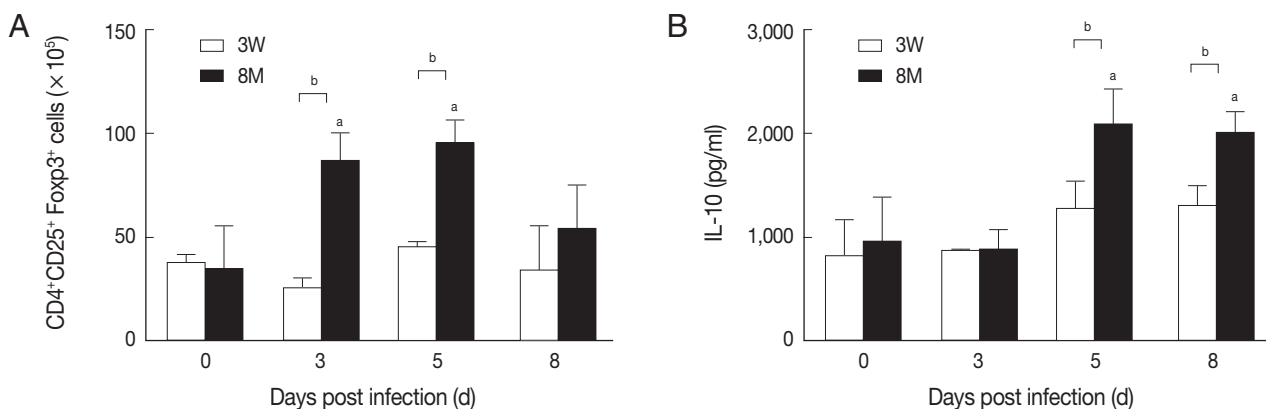
Fig. 3. Treg number and IL-10 levels in young and middle-aged mice with *P. berghei* ANKA infection. The number of CD4^{+}CD25^{+}Foxp3^{+} Tregs in total spleen T cell population was measured by flow cytometry for C57BL/6 mice after inoculation. ELISA was performed to detect the level of IL-10 in supernatants of cultured spleen cells from infected mice. Results are presented as the arithmetic mean of 9 mice per group±SE. [a]$P<0.05$ versus corresponding values for uninfected control mice (day 0 PI). [b]$P<0.05$ indicates the comparison between young and middle-aged mice.

fected young mice, whereas in adult mice on day 3 PI there was a significant increase ($P<0.05$) in the cell number up to 86.26×10^5 which then peaked at day 5 PI ($P<0.05$) with an absolute cell number of 94.76×10^5.

To elucidate the potential mechanism of immunosuppression by Tregs, we evaluated the Th1 inhibitory cytokine, IL-10, in supernatants of splenocytes from parasite-infected mice. There was a slight but insignificant increase in the level of IL-10 in young mice after parasitic infection. In contrast, the IL-10 level in middle-aged mice increased significantly ($P<0.05$) compared to both uninfected and young-infected mice and remained elevated from day 5 to day 8 PI (Fig. 3B). Thus, our data reveal a marked difference in the kinetics of Treg prolifer-

ation and the production of immunoregulatory cytokine, IL-10, between young susceptible and middle-aged resistant mice during *P. berghei* ANKA infection.

DISCUSSION

An appropriate immune response is critical in determining the outcome of malaria infection. Severe malaria, such as CM, is considered a complex multisystem disorder. The emergence of parasitemia and subsequent potent pro-inflammatory response are both essential for the occurrence of CM [26], and the development of CM is associated with high levels of inflammatory cytokines during malaria infection [27,28]. A mi-

nimal pro-inflammatory response is beneficial in limiting parasitemia, but an exacerbated host response invariably leads to tissue damage. Neutralization of the pro-inflammatory cytokine IFN-γ in vivo is protective against murine CM and its associated mortality. IFN-γ$^{-/-}$ [29] and IFN-γ receptor (IFN-γR)$^{-/-}$ [30] mice are resistant to CM. Although IFN-γ is thought to be part of the immune system response to malaria infection, an immunopathological role is also quite conceivable [31]. In humans, TNF-α is involved in the pathogenesis of human CM [32]. *P. berghei* ANKA-infected mice present with higher cerebral levels of TNF-α [33]. TNF-α is the principal cytokine mediator of CM in susceptible CBA/Ca mice [6]. Consistent with these findings, our experimental results showed that the levels of IFN-γ and TNF-α increased markedly after malaria parasite infection in young CM-susceptible mice. In particular, the TNF-α level was significantly higher in young mice than in middle-aged CM-resistant mice on day 3 PI, which indicates that an early excessive pro-inflammatory Th1 response with high levels of IFN-γ and TNF-α might be responsible for the susceptibility of young C57BL/6 mice to CM.

Several groups of T-cells, particularly the immunosuppressive Tregs, have been shown to play a critical role in balancing protective immune responses and immune-mediated pathology. In our study, Tregs expanded in middle-aged CM-resistant mice during infection with *P. berghei* ANKA as described previously [13,14], whereas in young CM-susceptible mice Tregs did not expand significantly. Thus, we demonstrated that this cell population has a regulatory role in the control of fatal pathogenesis. Moreover, the activation of Tregs accompanied by a high level of IL-10 was consistent with Tregs exerting their function in an IL-10-dependent manner [34,35]. IL-10 seems to have a host-protective role in murine malaria. In a susceptible mouse strain, administration of IL-10 gives some degree of protection against CM induced by *P. berghei* ANKA, while in a resistant strain, a neutralizing anti-IL-10 antibody leads to a significant incidence of cerebral complications [36]. In Vietnamese adults with severe malaria, plasma IL-10 levels were higher in those that died than in those who survived [37], but CM victims have even lower levels. IL-10 is pleiotropic but a relevant mechanism could be its ability to inhibit the production of cytokines, such as TNF-α [38], and it could also suppress excessive proinflammatory responses in human and experimental malaria [39]. Thus, the expansion of Tregs and the high level of IL-10, which counteracts the production of TNF-α, might limit the excessive pro-inflammatory Th1 response in

middle-aged mice, conferring resistance to CM, consistent with our previous study [23]. However, in young mice, the low level of Tregs and IL-10 may not control the early excessive pro-inflammatory Th1 response, causing the young mice to die from CM.

In summary, the significant finding in this report is the importance of Treg response during infection with *P. berghei* ANKA in young CM-susceptible and middle-aged CM-resistant C57BL/6 mice. Our results raise the possibility that IL-10-dependent Tregs play an immunosuppressive role in the establishment of early excessive pro-inflammatory Th1 immunity, leading to resistance to CM in the middle-aged mice.

ACKNOWLEDGMENTS

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