

L-Arginine Exacerbates Experimental Cerebral Malaria by Enhancing Pro-Inflammatory Responses

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L-Arginine (L-Arg), the substrate for nitric oxide (NO) synthase, has been used to treat malaria to reverse endothelial dysfunction in adults. However, the safety and efficacy of L-Arg remains unknown in malaria patients under the age of five, who are at the greatest risk of developing cerebral malaria (CM), a severe malaria complication. Here, we tested effects of L-Arg treatment on the outcomes of CM using a mouse model. Experimental cerebral malaria (ECM) was induced in female C57BL/6 mice infected with *Plasmodium berghei* ANKA, and L-Arg was administered either prophylactically or after parasite infection. Surprisingly, both types of L-Arg administration caused a decline in survival time and raised CM clinical scores. L-Arg treatment increased the population of CD4⁺T-bet⁺IFN- γ ⁺ Th1 cells and the activated macrophages (F4/80⁺CD36⁺) in the spleen. The levels of pro-inflammatory cytokines, IFN- γ and TNF- α , in splenocyte cultures were also increased by L-Arg treatment. The above changes were accompanied with a rise in the number of dendritic cells (DCs) and an increase in their maturation. However, L-Arg did not affect the population of regulatory T cells or the level of IL-10 in the spleen. Taken together, these data suggest that L-Arg may enhance the Th1 immune response, which is essential for a protective response in uncomplicated malaria but could be lethal in CM patients. Therefore, the prophylactic use of L-Arg to treat CM, based on the assumption that restoring the bioavailability of endothelial NO improves the outcome of CM, may need to be reconsidered especially for children.

Keywords: dendritic cells; L-Arginine; malaria; regulatory T cell; Th1 immune responses

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Introduction

Malaria, a mosquito borne parasite infection, is caused by several species of the genus *Plasmodium* (*P.*): *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*. According to the WHO world malaria report from 2013 (World Health Organization 2013), there were 207 million cases and 627,000 malaria deaths estimated globally in 2012. Infection with *P. falciparum* causes severe and sometimes fatal symptoms characterized by cerebral malaria (CM), multi-organ dysfunction, and non-cerebral severe malaria (NCSM) (Gupta et al. 1999; Walton et al. 2014). CM is one of the most severe complications and is a major cause of death after *P. falciparum* infection.

The host immune response significantly influences the outcome of malaria infection. An excessive immune response can result in immunopathology. It is generally

accepted that CM results from a combination of sequestering infected red blood cells in the cerebral blood vessels and a vigorous host pro-inflammatory response mediated by cytokines and effector cells (Hearn et al. 2000; Belnoue et al. 2002; Nitcheu et al. 2003; Franke-Fayard et al. 2005; Pais and Chatterjee 2005). The failure to establish a balance between the pro- and anti-inflammatory immune responses is likely one of the underlying causes of severe malaria (Walther et al. 2009). Therefore, an appropriate and effective immune response during malaria infection is essential for the host to eliminate this pathogen without causing additional damage.

Th1 cytokines play a critical role in controlling the early phase of malaria infection, but they are also associated with developing CM (Engwerda et al. 2005; Nie et al. 2007). A great deal of evidence about the inflammatory processes that contribute to the development of CM has

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been provided by the *Plasmodium berghei* ANKA (*Pb* ANKA) model (de Souza and Riley 2002; Miller et al. 2002; Schofield and Grau 2005; Hansen 2012). We have previously shown that regulatory T cells (Treg) prevent the development of CM by modifying the pro-inflammatory response in *Pb* ANKA-infected C57BL/6 mice (Wu et al. 2010).

L-Arginine (L-Arg) is considered an essential amino acid for infants, but not for adults. Recent studies suggested that L-Arg is crucial for the function of host immune cells. L-Arg deprivation induces T cell hypo-responsiveness, defined as a profound reduction in T cell proliferation and reduced CD3 ζ chain expression (Rodriguez et al. 2002, 2003; Zea et al. 2004, 2005; Kropf et al. 2007). L-Arg deficiency is also correlated with a dramatic reduction in B-cell maturation and a significant decrease in serum IgM, due to an impaired transition from pro- to pre-B cells (de Jonge et al. 2002). Macrophage activation is also dependent on L-Arg (Yeramian et al. 2006), which suggests L-Arg might contribute to the production of nitric oxide (NO). Abnormal NO production is believed to play a significant role in the pathogenesis of severe malaria (Yeo et al. 2008a). Low serum levels of NO have been reported in individuals with malaria, and the lowest levels observed in children who died from CM (Anstey et al. 1996). L-Arg, the substrate for NO synthase, has been used to treat malaria. Phase I trials have shown that L-Arg is safe and well tolerated in adults with moderate-to-severe malaria, and arginine infusion has been associated improving NO bioavailability leading to recovery of endothelial function (Yeo et al. 2008a). However, little is known about the safety and efficacy of L-Arg supplementation in malaria patients under the age of five who are considered to have the greatest risk of developing severe malaria complications, like CM.

To further characterize the role of L-Arg in developing CM, we examined the production of pro-inflammatory cytokines and changes to the functions of effector cells including T cells, macrophages, and dendritic cells (DCs) in an experimental CM model supplemented with L-Arg. We demonstrated that, although L-Arg treatment increased NO production, it also enhanced the Th1 pro-inflammatory immune response, leading to accelerated CM development. Our findings suggest that prophylactic strategies based on NO restoration to improve the outcome of experimental CM may be more complicated than originally envisioned.

Materials and Methods

Mice, parasites, and experimental infection

Female 6-8-week-old C57BL/6 mice were purchased from the Center of Zoology, Chinese Academy of Sciences (Shanghai branch). *Pb* ANKA was kindly provided by Dr. Motomi Torii (Department of Molecular Parasitology, Ehime University Graduate School of Medicine, Ehime, Japan). Mice were infected by intraperitoneal (i.p.) injection of 1×10^6 *Pb* ANKA parasitized erythrocytes from C57BL/6 mice. Parasitemia was monitored by examining Giemsa-stained, thin (tail) blood smears under a light microscope (Slade and Langhorne

1989). Mortality was monitored daily. A clinical experimental cerebral malaria (ECM) score was defined based on the presence of the following signs: ruffled fur, hunching, wobbly gait, limb paralysis, convulsions, and coma. For each sign present the mouse was given a score of 1. Animals with scores ≥ 4 were considered to have severe ECM. Animal experiments in this study were approved by the Animal Ethics Committee of China Medical University.

L-Arg treatment

L-Arg was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in normal saline before use. L-Arg (1.5 mg/g) was given orally to two groups of mice either before (Pre-L-Arg) or after *Pb* ANKA infection (Pro-L-Arg). In the Pre-L-Arg group, mice received L-Arg once a day for seven days until they were infected with malaria parasites. While in the Pro-L-Arg group, L-Arg was administered from the day of malaria infection until the mice died or were cured. An equivalent volume of normal saline was used for the control group.

Splenocyte preparation and culture

Splenocyte culture was performed as previously described (Wang et al. 2009). Briefly, the spleens from normal and infected mice were removed aseptically and pressed through a sterile fine-wire mesh with 10 mL of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 25 mM Hepes, 0.12% gentamicin, and 2 mM glutamine. The resulting cell suspensions were pelleted by centrifuging at 350 g for 10 min. The erythrocytes in the splenocyte suspension were lysed with cold 0.17 M NH₄Cl and the remaining cells were washed twice with fresh medium. After the erythrocytes had been lysed, the splenocytes were cultured (500 μ L/well) into 24-well flat-bottom tissue culture plates (Falcon) in triplicate and incubated for 48 h at 37°C in a humidified 5% CO₂ incubator. The supernatants were collected and stored at -80°C until they were used to detect cytokines and NO levels.

Flow cytometry analysis

Mice were sacrificed at the indicated time points after infection and splenocytes were isolated for flow cytometry analysis. The viability of the cells was determined by Trypan blue exclusion test and only samples with viability > 98% were used for further staining. The subsets of spleen cells were defined as: CD11c⁺CD11b⁺ myeloid DCs, CD11c⁺CD45R/B220⁺ plasmacytoid DCs, F4/80⁺CD36⁺ macrophages, CD4⁺T-bet⁺IFN- γ ⁺ T cells, and CD4⁺CD25⁺Foxp3⁺ Treg. Mature CD11c⁺DCs were identified by the expression of MHCII, CD86, and TLR9. Unless otherwise indicated, antibodies were purchased from BD Pharmingen. The following antibodies were used: FITC-conjugated anti-CD11c (clone HL-3), PE-conjugated anti-CD11b (clone M1/70), PerCP-conjugated anti-B220 (clone RA3-6B2), PE-conjugated anti-MHCII (clone M5/114.15.2), PE-conjugated anti-CD86 (clone GL1), biotinylated anti-TLR9 (clone 5G5, purchased from Hycult biotech), PE-conjugated streptavidin (purchased from Biolegend), APC-conjugated anti-IFN- γ (XMG1.2), FITC-conjugated anti-F4/80 (clone BMB), PE-conjugated anti-CD36 (clone 72-1, purchased from eBioscience), FITC-conjugated anti-CD4 (clone GK1.5), anti-T-bet-PE (clone eBio4B10, purchased from eBioscience), PE-conjugated anti-CD25 (clone PC61), and APC-conjugated anti-Foxp3 (clone FJK16s, purchased from eBioscience).

Intracellular staining

To measure the intracellular level of IFN- γ , 1×10^7 fresh splenocytes were stimulated in 12 -well plates with phorbol myristate acetate (50 ng/mL) and ionomycin (0.5 μ g/mL). After 2 hours, Golgi stop (2 μ M) was added to block cytokine export. After an additional 4 hours of incubation (6 hours total), the cells were surface stained with anti-CD4-FITC. The cells were then fixed, permeabilized, and stained with anti-T-bet-PE and anti-IFN- γ -APC. Finally, the cells were washed with $1 \times$ Perm Wash (BD Bioscience, UK), re-suspended in FACS buffer, and collected on a FACS Calibur cytometer. Data analysis was performed using FlowJo (TreeStar Inc., Ashland, OR).

Determining cytokine concentration by ELISA

Levels of IFN- γ , TNF- α and IL-10 were measured using commercial enzyme linked immunosorbent assay (ELISA) kits according to the manufacturer's protocols (R&D Systems, Minneapolis, MN). The OD values were measured in a microplate reader at 450 nm. The concentration of cytokines in each sample was calculated using a standard curve generated from recombinant cytokines.

Determining nitrite (NO_2^-) concentration

The concentration of NO_2^- was determined in cell supernatants using the Griess reaction (Wang et al. 2009). Briefly, 100 μ L of the culture supernatant from freshly isolated, unstimulated splenocytes was incubated with 100 μ L of Griess reagent for 10 min at room temperature. The NO_2^- concentration was determined by measuring the optical density at 550 nm (A550) in reference to the A550 of a standard NaNO_2 solution.

Statistical analysis

Data are presented as the means \pm standard errors of the means (SEM). Statistically significant differences between groups were determined using the *t* test. Time-to-event data were analyzed using the Kaplan-Meier (K-M) approach to survival analysis (SPSS 17.0). A value of $p < 0.05$ was considered significant.

Results

L-Arg supplementation accelerates the death of *Pb* ANKA-infected C57BL/6 mice while simultaneously inhibiting parasitemia

To determine the effects of L-Arg supplementation on

the outcome of ECM, C57BL/6 mice were treated with L-Arg before or after *Pb* ANKA infection. Parasitemia (Fig. 1A) and survival rate (Fig. 1B) were monitored after infection. Parasitemia was similar in the two L-Arg-treated groups, and parasitemia in both L-Arg groups was lower than the control group. On day 7 after infection, parasitemia in the mice received L-Arg pre-infection (Pre-L-Arg group) was significantly lower than that of the mice given normal saline ($p < 0.01$). However, despite reduced parasitemia, mice in the L-Arg-treated groups died at days 6-8 after infection, which was significantly earlier than the control group (days 8-11 after infection; $p < 0.05$). Most of dying mice exhibited ruffled fur, hunching, wobbly gait, and convulsions. The clinical ECM scores were higher in the L-Arg-treated groups than the control group (Fig. 1C). Taken together, these data suggest that L-Arg-treated mice died from immune mediated pathology rather than from anemia.

L-Arg enhances the pro-inflammatory immune response in *Pb* ANKA-infected C57BL/6 mice

To elucidate the immunological mechanisms that could contribute to more rapid death of *Pb* ANKA-infected C57BL/6 mice supplemented with L-Arg, we investigated changes in the Th1 response, macrophage activation, and production of inflammatory cytokines in these mice. L-Arg treatment significantly increased the frequency and number of IFN- γ -secreting Th1 cells on day 3 after infection, compared with control group (Fig. 2A-C). Concurrently, on days 3 and 5 after infection, the levels of IFN- γ (Fig. 2D) and TNF- α (Fig. 2E) were significantly increased in the L-Arg-treated groups compared with the control group. We next assessed the number and function of macrophages that related to pro-inflammatory reaction in the spleen of *Pb* ANKA-infected mice. We found that mice from the L-Arg treated groups had significantly more F4/80⁺CD36⁺ macrophages (Fig. 3A-C) in the spleen on days 3 and 5 after infection. As it was reported that splenic macrophages produced high levels of NO in the mouse model of blood-stage malaria (Ahvazi et al. 1995), we also measured the level of

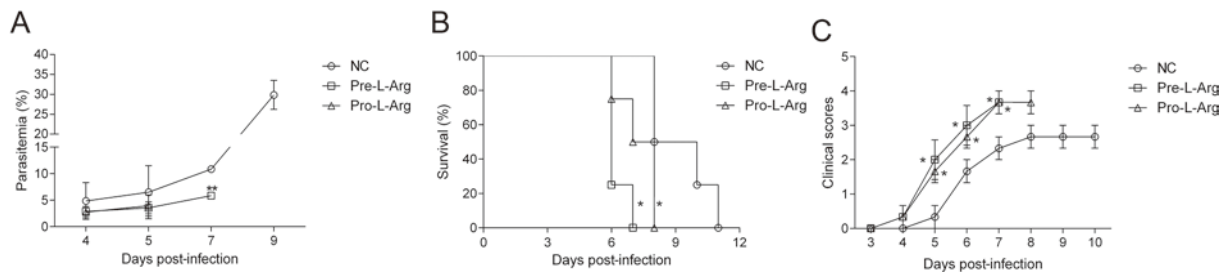


Fig. 1. L-Arg decreases the survival rate of *Pb* ANKA-infected mice.

(A) Parasitemia was determined by evaluating Giemsa-stained thin blood films under a light microscope. (B) Survival rate were monitored daily, and the data from three independent experiments were combined ($n = 12$). (C) Clinical scores for the severity of ECM were assessed daily based on the number of symptoms each mouse displayed. In A and C, values represent the mean \pm SEM and results are the representative of three independent experiments each containing 3-5 mice per group. Symbols * and ** indicate $p < 0.05$ and $p < 0.01$, respectively, compared with the control (NC) group.

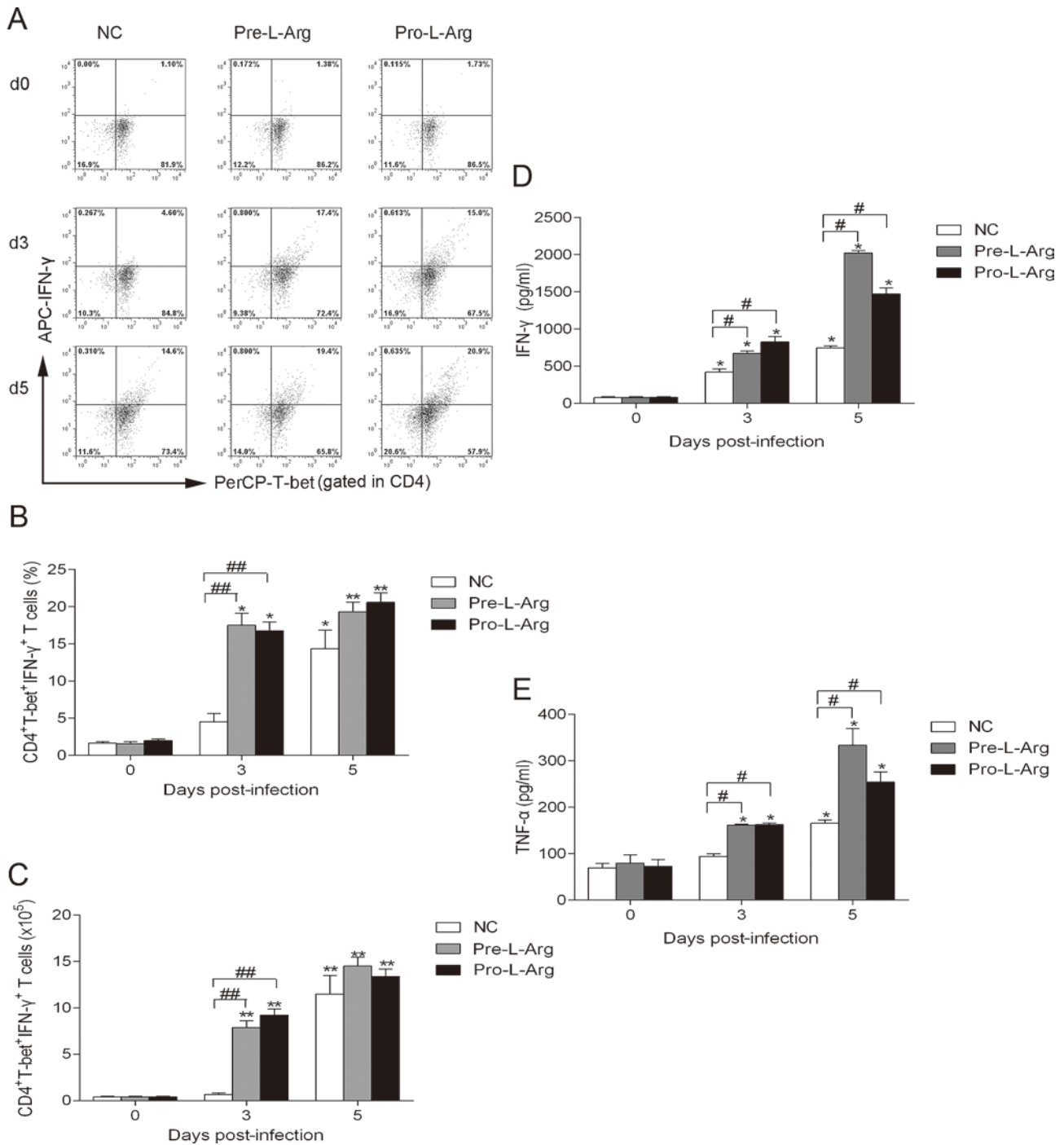


Fig. 2. L-Arg enhances Th1 immune responses in *Pb* ANKA-infected mice.

CD4⁺T-bet⁺IFN- γ ⁺ Th1 cells (gated on CD4⁺) were analyzed by flow cytometry and displayed in dot plot (A). Their percentage and absolute number are shown in B and C. Concentrations of cytokine IFN- γ (D) and TNF- α (E) in the culture supernatant of spleen cells were determined by ELISA. Values represent the mean \pm SEM ($n = 3$ mice per group). Results are representative of three independent experiments. Symbols * and ** indicate statistically significant differences ($p < 0.05$ and $p < 0.01$, respectively) between infected groups and uninfected groups; # and ## indicate statistically significant differences ($p < 0.05$ and $p < 0.01$, respectively) between L-Arg-treated mice and normal saline-treated mice.

NO in the culture supernatant of splenocytes. As expected, splenocytes from mice in the L-Arg groups showed much higher NO production than that from the control group on days 3 and 5 after infection (Fig. 3D). Thus, there was an

increased pro-inflammatory response in L-Arg-supplemented C57BL/6 mice infected with *Pb* ANKA, which might contribute to the development of CM.

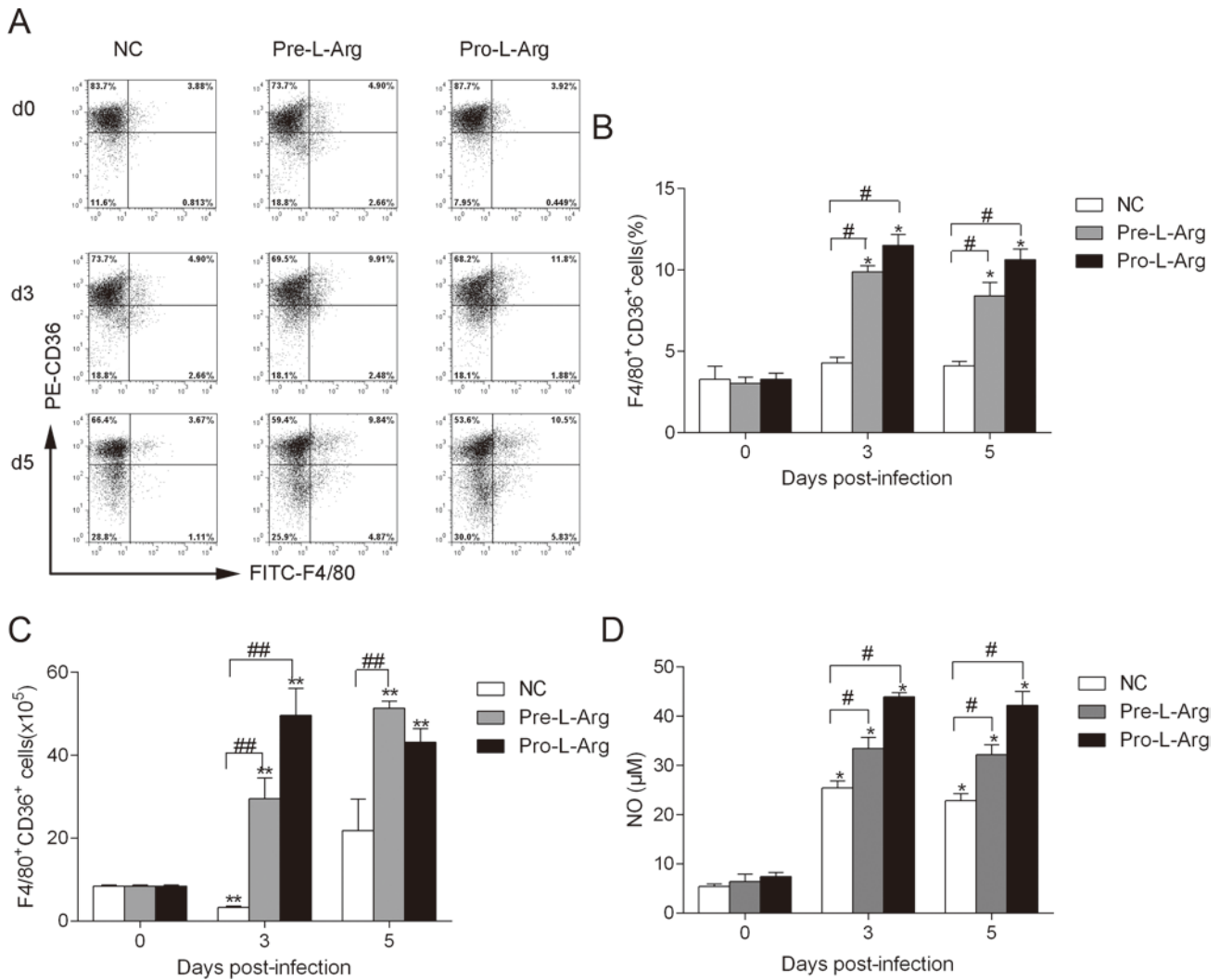


Fig. 3. L-Arg promotes the expansion and activation of macrophages in *Pb* ANKA-infected mice.

The population of F4/80⁺ CD36⁺ cell in splenocytes was analyzed by flow cytometry and displayed in dot plot (A). Their percentage and absolute number are shown in B and C. The level of NO₂⁻ in the supernatants of cultured splenocytes was determined by Griess reaction (D). Values represent the mean ± SEM ($n = 3$ mice per group). Results are representative of three independent experiments. Symbols * and ** indicate statistically significant differences ($p < 0.05$ and $p < 0.01$, respectively) between infected groups and uninfected groups; # and ## indicate statistically significant differences ($p < 0.05$ and $p < 0.01$, respectively) between L-Arg-treated mice and normal saline-treated mice.

L-Arg stimulates DC activation and proliferation in *Pb* ANKA-infected C57BL/6 mice

DCs play a critical role in the activation and polarization of naive Th cells. To determine the immunomodulatory effects of L-Arg on DCs, we examined the frequency and maturation of DCs from *Pb* ANKA-infected mice. The percentage and numbers of myeloid DCs (Fig. 4A-C) and plasmacytoid DCs (Fig. 4D-F) in L-Arg-treated groups were significantly higher than that in the control groups on days 3 and 5 after infection ($p < 0.05$ and $p < 0.01$, respectively). We next assessed the expression of MHCII and CD86 on splenic CD11c⁺ DCs, which are essential for inducing a T-cell response. As expected, L-Arg significantly enhanced the percentages and numbers of CD11c⁺MHCII⁺ DCs (Fig. 5A-C) and CD11c⁺CD86⁺ DCs (Fig. 5D-F) at day 3 after infection ($p < 0.05$) and day 5

after infection ($p < 0.05$). As the pattern recognition molecules toll-like receptors (TLRs) have been known to be involved in the recognition of protozoan parasites (Iwasaki and Medzhitov 2004), we quantified the expression of TLR9 on DCs. As expected, the percentages and number of CD11c⁺TLR9⁺ DCs was significantly increased in L-Arg treated groups compared with control group (Fig. 5G-I).

L-Arg has no effect on the number of Tregs or the level of IL-10 in *Pb* ANKA-infected C57BL/6 mice

CD4⁺CD25⁺Foxp3⁺ Tregs have an important role in regulating pro-inflammatory responses in rodent malaria models during early infection. The potential regulatory mechanisms are associated with the induction of IL-10 (Chen et al. 2009). To further investigate the effects of L-Arg supplementation on Tregs and IL-10 production, we

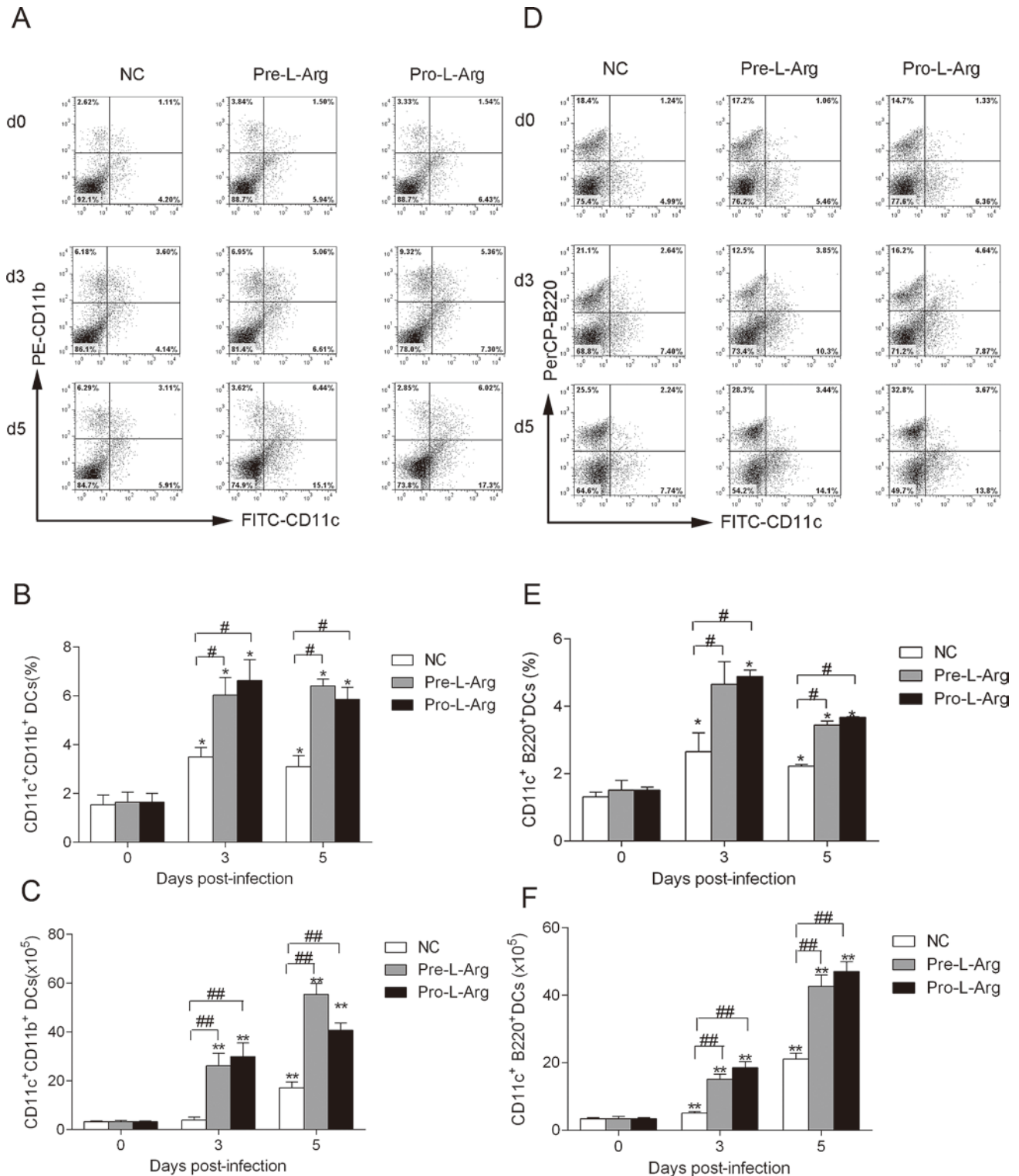


Fig. 4. L-Arg promotes the proliferation of DCs' subsets in *Pb* ANKA-infected mice.

The percentage of CD11c⁺CD11b⁺ DCs (A and B) and CD11c⁺B220⁺ DCs (D and E) in the spleen cells was evaluated by flow cytometry and shown in dot plot and bar graph. The absolute number of these cells was calculated and shown (C and F). Data are presented as the mean \pm SEM ($n = 3$ mice per group). Results are representative of three independent experiments. Symbols * and ** indicate statistically significant differences ($p < 0.05$ and $p < 0.01$, respectively) between infected groups and uninfected groups; # and ## indicate statistically significant differences ($p < 0.05$ and $p < 0.01$, respectively) between L-Arg-treated mice and normal saline-treated mice.

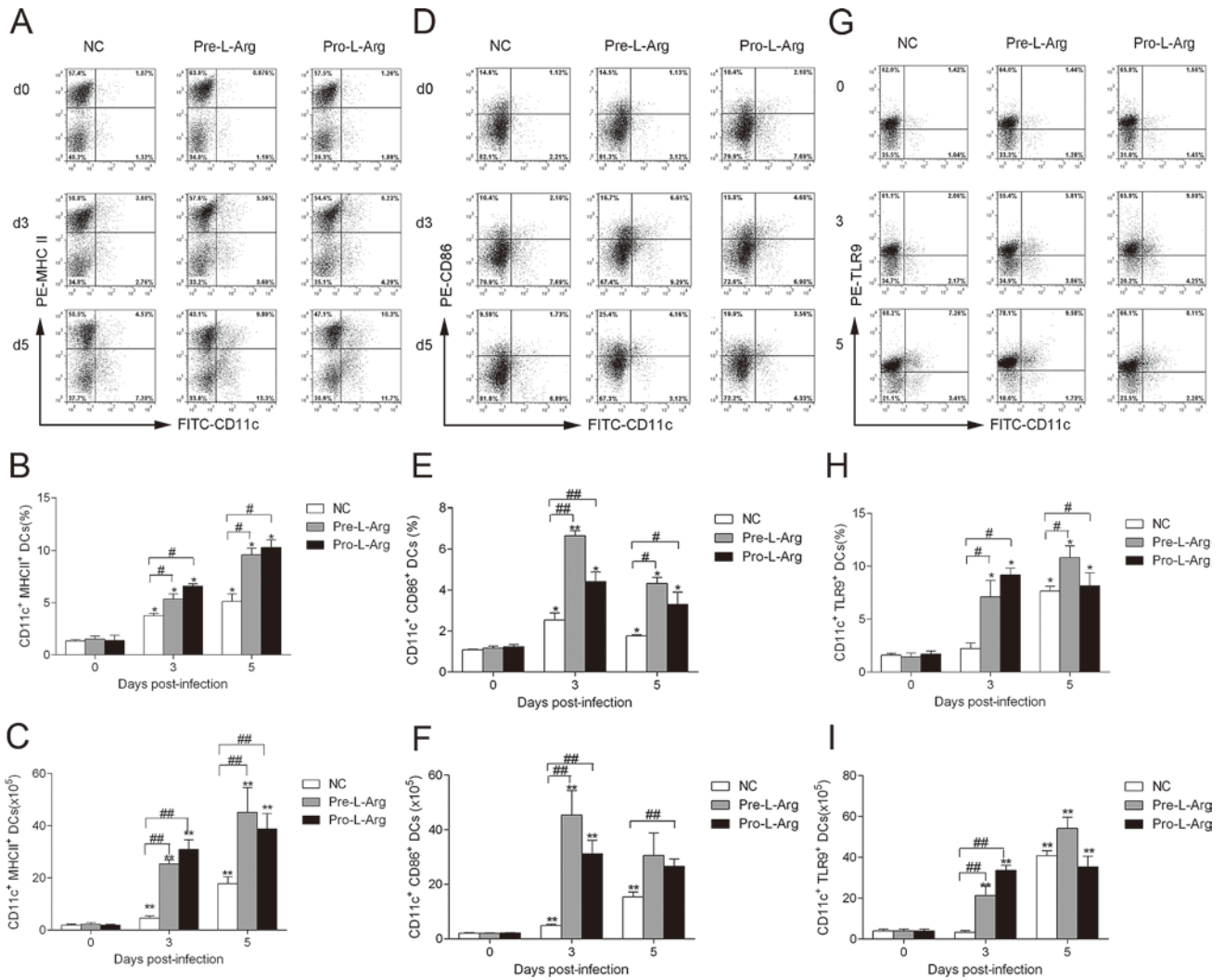


Fig. 5. L-Arg promotes the expression of DCs' phenotype in *Pb* ANKA-infected mice. The expression of MHCII (A), CD86 (D) and TLR9 (G) on splenic CD11c⁺ DCs was evaluated by flow cytometry and displayed in dot plot. The percentage and absolute number of CD11c⁺MHCII⁺ (B and C) CD11c⁺CD86⁺ (E and F) and CD11c⁺TLR9⁺ DCs (H and I) was shown in bar graph. Data is presented as the mean ± SEM (*n* = 3 mice per group). Results are representative of three independent experiments. Symbols * and ** indicate statistically significant differences (*p* < 0.05 and *p* < 0.01, respectively) between infected groups and uninfected groups; # and ## indicate statistically significant differences (*p* < 0.05 and *p* < 0.01, respectively) between L-Arg-treated mice and normal saline-treated mice.

determined the percentage and number of Tregs as well as levels of IL-10 produced by splenocytes in *Pb* ANKA-infected C57BL/6 mice. As shown in Fig. 6, the percentage and number of Tregs increased in the control, Pre-L-Arg, and Pro-L-Arg groups after parasite infection. However, there was no significant difference between the L-Arg treated groups and the control group (Fig. 6A-C). Similar results were observed regarding the production of splenic IL-10 (Fig. 6D). These data suggested that the increased Th1 response in the ECM model supplemented with L-Arg was not due to deficient Tregs or IL-10.

Discussion

In this study, we demonstrate for the first time that oral L-Arg treatment, regardless of whether it is pre- or post-

infection, significantly decreases the survival time of mice with ECM probably by enhancing Th1 immune responses. Our findings provide important insights into the usage of L-Arg in areas where malaria is endemic.

L-Arg is a conditionally essential amino acid for adults and plays a critical role in regulating the immune response (Tapiero et al. 2002; Bronte and Zanovello 2005). L-Arg is important primarily because of its role as a precursor for NO synthesis. NO is a free radical molecule that is synthesized by NO synthase (NOS) in all mammalian cells, but excessive NO production can be pathogenic in both acute and chronic models of inflammation (Salvemini and Marino 1998). In case of adults who do not develop CM, have a premunitive status, or were previously infected with *P. falciparum*, L-Arg is a potential adjunctive therapy for clinical

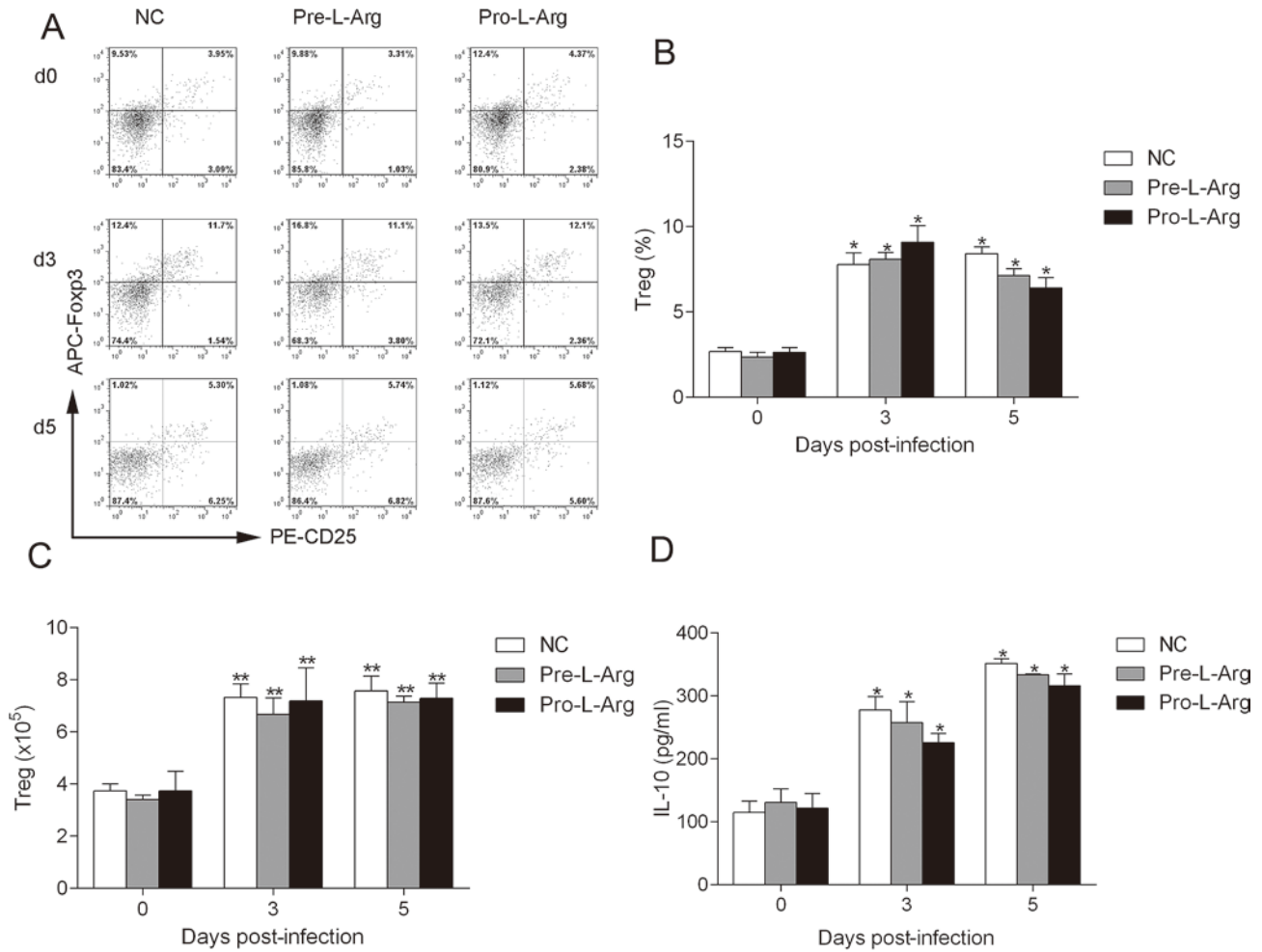


Fig. 6. L-Arg has no effect on Tregs in *Pb* ANKA-infected mice.

The percentage of Tregs (gated on CD4⁺) in the spleen was evaluated by flow cytometry and shown in dot plot (A) and bar graph (B) at 0, 3, and 5 days after infection. The absolute number of these cells is shown (C). The level of IL-10 in the culture supernatant of splenocytes was measured with ELISA (D). Data are presented as the mean \pm SEM ($n = 3$ mice per group). Results are representative of three independent experiments. Symbols * and ** indicate statistically significant differences ($p < 0.05$ and $p < 0.01$, respectively) between infected groups and uninfected groups.

recovery from severe malaria (Yeo et al. 2007). We previously showed that L-Arg potentiated Th1 immune response during *Plasmodium yoelii* (*Py*) 17XL infection, which supported that L-Arg was an effective immunomodulator for uncomplicated mouse malaria (Zhu et al. 2012). However, enhanced pro-inflammatory immune response could result in immunopathology in malaria patients, especially in children under five, who develop CM more frequently than adults during *P. falciparum* infection. Therefore, we wanted to address whether L-Arg supplementation was an appropriate adjunct therapy for young children in malaria endemic area. In the current study, we observed that L-Arg treatment accelerated the death of mice with ECM. We also found that L-Arg increased Th1 response to *Pb* ANKA infection as it was previously shown in *Py*17XL-infected mice. Whereas the elevated Th1 response contributed to the survival of mice during *Py*17XL infection, it was associated with aggravation of CM in mice infected with *Pb* ANKA. Our findings suggested that, although L-Arg sup-

plementation is safe in adult malaria patients, it might be detrimental for young children who are at more risk for CM during *P. falciparum* infection.

Clinically CM is characterized by convulsions, seizures, and coma. Many studies have shown that parasitemia and the subsequent potent pro-inflammatory response are essential for developing CM (Walther et al. 2009). In a commonly used murine CM model, the *Pb* ANKA-infected C57BL/6 mice, the development of CM is associated with high levels of inflammatory mediators such as IFN- γ , TNF- α , and NO during infection (Hunt and Grau 2003; Engwerda et al. 2005; Jain et al. 2008; Edgerton et al. 2009; Wang et al. 2009). In agreement with these work, here we found that splenocytes producing pro-inflammatory mediators, such as IFN- γ -secreting Th1 cells and F4/80⁺CD36⁺ cells, were both increased in spleen from *Pb* ANKA-infected mice treated with L-Arg. In addition, the levels of IFN- γ , TNF- α , and NO were also significantly raised by L-Arg treatment during infection. It needs to be mentioned that

Th1 immune response was not enhanced in Pre-L-Arg group on day 0 post infection. This implies that prophylactic supplementation of L-Arg for one week had no direct effects on the function of resting T cells. The enhancement of Th1 response by L-Arg may largely be secondary to the modification of DCs' function. Despite that, our data indicated that L-Arg potentiated Th1 response and the production of pro-inflammatory cytokines in *Pb* ANKA-infected C57BL/6 mice, which may contribute to the aggravation of CM.

We also noticed that L-Arg treatment reduced the level of parasitemia while increasing Th1 response in the *Pb* ANKA-infected C57BL/6 mice. This result is reasonable since Th1 response plays a central role in controlling parasitemia. However, there is the possibility that L-Arg acts directly on the viability of malaria parasite. It was reported that *P. falciparum* parasites under the pressure of S-nitroso-N-acetyl-penicillamine (SNAP), the NO donor, died by autophagic-like cell death (Totino et al. 2008). Another study showed that NO production increased in *P. falciparum* parasites incubated with L-Arg in vitro (Rey et al. 2014). These results suggest that L-Arg supplementation may have a direct effect on the viability of malaria parasites in vivo, and more work needs to be done to prove it.

DCs are heterogeneous antigen presenting cells that have a key role in initiating and regulating cell-mediated immune responses (Banchereau et al. 2000). DCs are required to initiate the malaria parasite-specific CD4⁺ T cell response, and subsequently CM (deWalick et al. 2007). In this study, we showed that L-Arg treatment increased the number of DCs and promoted their maturation in spleen of *Pb* ANKA-infected mice. These changes could have contributed to enhancing the Th1 response in mice treated with L-Arg in our experiment. However, how L-Arg treatment influence DCs' number and function is not clear. It is known that DCs' function is regulated by multiple tissue factors, such as IFN- γ and CD40 ligand produced by activated gamma delta T cells during *Plasmodium* infection (Yanez et al. 1999; Inoue et al. 2012). Thus, L-Arg treatment may affect DCs' number and function via both direct and indirect ways. Studies to understand how supplementing L-Arg might affect DC function would be worth pursuing in the future studies.

TLRs are critical components of the innate immune system and recognize conserved microbial products including lipids, proteins, and nucleic acids (Akira et al. 2006). A role for TLRs in the pathogenesis of malaria has been proposed by several different studies. Although the data is conflicting, there is a growing body of literature that supports viewing TLRs as central regulators of pro-inflammatory responses in both rodent and human malaria (Ishii et al. 2005; Zhu et al. 2005; Coban et al. 2007; Parroche et al. 2007). TLR9 is thought to play an essential role in CM pathogenesis by helping recruit immune cells into the brain, or in the case of severe malaria, for its role in inducing Tregs and/or synergy with IFN- γ signaling (Coban et al.

2007; Hisaeda et al. 2008). DCs from TLR9 (-/-) and MyD88 (-/-) mice produced significantly lower levels of pro-inflammatory cytokines, but higher levels of anti-inflammatory cytokines, than wild-type mice (Gowda et al. 2012). Consistent with previous reports, TLR9 expression on DCs from L-Arg treated mice was significantly increased in this study, and was associated with elevated pro-inflammatory cytokine levels. However, the mechanism underlying L-Arg regulation of TLR9 expression remains unclear.

Tregs are a T-cell subset that has different immunoregulatory effects from Th1 and Th2 cells. Tregs inhibit the Th1 response by modifying DC function, producing IL-10, and inducing CD4⁺ T cell apoptosis (Slade and Langhorne 1989; Zheng et al. 2009), which facilitates immune evasion by the parasite (Hisaeda et al. 2004). Previous studies have shown that Tregs contributed to determining the incidence and outcome of CM in *Pb* ANKA-infected mice by altering the balance of pro- (IFN- γ , TNF- α , IL-6, IL-17, and NO) and anti-inflammatory (IL-10) responses (Lopansri et al. 2003; Amante et al. 2007; Yeo et al. 2008b). Therefore, we assessed the effect of L-Arg on the percentage of Tregs and the production of the anti-inflammatory cytokine IL-10. In the *Pb* ANKA/C57BL/6 model, L-Arg affected neither the percentage of Tregs nor IL-10 production. This suggested that Tregs were not likely contributed to the enhanced Th1 response in the L-Arg supplemented CM model.

As NO is a potential target for therapy of severe malaria, a variety of strategies has been developed to improve NO bioavailability. Difference in NO donors, dosage and administration route resulted in controversial studies (Martins et al. 2012). In our experiments, oral L-Arg treatment accelerates the death of mice in the *Pb* ANKA/C57BL/6 ECM model. This effect is associated with enhanced Th1 responses and activation of DCs, but did not involve Tregs. We wish to emphasize that in this study L-Arg treatment enhanced the Th1 immune response. Although L-Arg has a protective role in non-CM patients, it may contribute to a lethal outcome in CM patients. Therefore, L-Arg should be used with caution in malaria patients of different ages in the endemic areas. Although supplementing with L-Arg has generally been safe, our study provides additional information about potentially life-threatening applications of L-Arg in CM patients in malaria-endemic areas.

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Conflict of Interest

The authors declare no conflict of interest.

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