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EXPERIMENTAL CEREBRAL MALARIA ALTERS BLOOD LIPID LEVELS DURING PATHOGENESIS

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KEY WORDS

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ABSTRACT

Malaria infection threatens millions of people worldwide. Sequestering of *Plasmodium*-infected erythrocytes within the blood vessels of the brain may lead to a more severe form of disease called cerebral malaria (CM), which is difficult to diagnose and treat. Here we used C57BL/6 mice to establish a model of experimental CM (ECM). Comparing the dosage dependence of ECM induction, we found that inoculation with 1×10^3 parasitized erythrocytes had higher efficiency at establishing ECM than 1×10^6 parasitized erythrocytes. However, the percentage of ECM varied in different experimental batches. Infected mice that developed ECM had elevated serum levels of total cholesterol and decreased serum levels of high-density lipoprotein and low-density lipoprotein cholesterol. In addition, ECM mice exhibited liver and kidney dysfunction. ECM induced by low dose inoculation requires additional verification for efficiency. Biochemical analysis of ECM mice revealed characteristic blood lipid levels. These findings provide new clues for the diagnosis and mechanistic probing of CM pathogenesis.

Malaria is a contagious parasitic disease, infecting 4.45 million people worldwide in 2016. Most of the victims are children in sub-Saharan Africa (WHO, 2017). Cerebral malaria (CM) is the most serious complication of *Plasmodium falciparum* infection and the most common cause of death, evolving rapidly in the absence of appropriate treatment. The symptoms of CM include headache, seizures, coma, and neurological symptoms. The early signs of CM are not typical and pathognomonic and are difficult to discriminate from encephalitis, meningitis, and febrile convulsions (de Souza et al., 2010). This pathogenic characteristic of CM impedes immediate medical intervention. Patients suffering from CM need to be treated with parenteral antimalarial therapy, and even so the cure rate is unsatisfactory. Adjunctive therapies such as neuroprotective drugs and immuno-modulators also may be applied. Even if CM patients are effectively treated and survive, they may develop long-term neurological and neurocognitive dysfunctions, leading to undesirable societal consequences (Zimmerman and Castro-Faria-Neto, 2010).

The pathogenic mechanisms of human cerebral malaria (HCM) are complicated and elusive. Previous reports suggest that cytoadherence and inflammation might be major factors in HCM (Storm and Craig, 2014). Cytoadherence of parasitized red blood cells (pRBCs) to endothelial cells leads to sequestration and microvasculature occlusion associated with perivascular hemorrhages and microvascular congestion (Strangward et al., 2017). Another pathophysiological change in CM involves the imbalance of pro- and anti-inflammatory responses upon parasite infection (Dunst et al., 2017). Inflammatory mediators, including chemokines and cytokines, induce and exacerbate local inflammation in the brain, prompting the pathogenesis of CM (Idro et al., 2010). Additionally, endothelial dysfunction, neuron and glia apoptosis, and blood-brain barrier (BBB) damage also refer to CM (Storm and Craig, 2014).

Since the utilization of a human brain pre-mortem to investigate CM pathogenesis is unpractical, an experimental mouse model of cerebral malaria (ECM) has been developed. Several strains of mice are susceptible to CM, including C57BL/6J, C57BL/6N, CBA/ca, and Swiss (Engwerda et al., 2005). Rodent *Plasmodium berghei* and *Plasmodium yoelii* have been widely used in CM models, especially *P. berghei* ANKA (*Pb*A). The pathogenesis of ECM and HCM has been proposed to be different, primarily due to the lack of parasite sequestration in the brain in ECM (Franke-Fayard et al., 2005). A histopathological study demonstrated that pRBCs accumulate in the brain capillaries in ECM. Although the patterns of pRBC-dependent microvascular obstruction between ECM and HCM are distinct, the change in cerebral blood flow induced by pRBC accumulation is expected to be similar

(Strangward et al., 2017). Therefore, murine ECM is a useful tool for studying the pathogenesis of HCM.

Overall, CM is difficult to diagnose and treat because of its acute onset, rapid progression, and uncertain pathogenesis. It is necessary to identify biomarkers or therapeutic targets in ECM models. In the current study, we constructed ECM models using different inoculum doses and found that, in general, inoculation with 1×10^3 pRBCs has higher efficiency in establishing ECM than 1×10^6 pRBCs. The incidence of ECM among different experimental batches varied. Biochemical analysis revealed characteristic blood lipid levels in ECM compared to non-cerebral malaria (NCM). In addition, ECM mice exhibited liver and kidney dysfunction. These findings provide new clues for the diagnosis and mechanistic probing of the pathogenesis of CM.

MATERIALS AND METHODS

Mice and ethics statement

Female C57BL/6 mice aged 6–8 wk (weighing 20–25 g) or 8–10 wk (weighing 25–30 g) were purchased from Laboratory Animal Center of Academy of Military Medical Sciences (Beijing, China). All animals were maintained under standard conditions according to the guidelines of the Medical Research Animal Care Committee of Tianjin Medical University (TMU). This project was approved and monitored by the Medical Research Animal Ethics Committee of TMU.

Parasite inoculation

Plasmodium berghei ANKA lines were used in all experiments. Blood-stage parasites of *P. berghei* ANKA were stored as a stabilate in liquid nitrogen. The parasites were cultivated by passage through C57BL/6 mice. Experimental infection of mice was started with the intraperitoneal (IP) or intravenous (IV) injection of 1×10^3 or 1×10^6 pRBCs. Parasitemia was monitored in the infected mice by light microscope examination of Giemsa-stained thin smears of tail blood.

Cerebral malaria assessment

Infected mice were monitored daily after day 5 post-infection (p.i.) and evaluated for symptoms of ECM. Clinical scale assessment of ECM was used to estimate whether the mice had developed CM (de Oca et al., 2013). Mice were considered to have CM if they presented neurological symptoms (i.e., stage 4–5 of the criterion mentioned above), such as reduced responsiveness to stimulation, ataxia, respiratory distress, prostration, paralysis, and convulsions. A few mice died shortly after CM manifestation, and these mice were classified as having CM.

Blood-brain barrier integrity

To evaluate the integrity of the BBB, 200 µl of 2% Evans Blue in PBS solution (pH 7.2) was injected intravenously into mice with CM symptoms. The mice were euthanized and perfused with 40 ml PBS 1 hr after the injection. The brain of each mouse was dissected and weighed, then immersed in 1 ml formamide (Sangon Biotech, Shanghai, China) for 48 hr at 37 C to extract the Evans Blue dye. The optical density (OD) of the solution was detected by a spectrophotometer at 630 nm. Based on a standard curve, Evans Blue extravasation was calculated as micrograms Evans Blue dye per gram of brain tissue.

Serum biochemical indices

Blood samples were collected by retro-orbital bleeding. The blood was allowed to clot for 60 min and separated by centrifugation at 4 C (2,700 *g*) for 10 min. The supernatant was collected and stored at −80 C. Serum samples were processed in 1 batch to detect biochemical indices using a Roche P800 Clinical Biochemistry Analyzer (F. Hoffmann-La Roche Ltd., Basel, Switzerland), including alanine aminotransferase (ALT), aspartate transaminase (AST), blood urea nitrogen (BUN), creatinine (Cr), total triglycerides (TGs), total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c), and very low-density lipoprotein cholesterol (VLDL-c).

Statistical analysis

Data are presented as mean ± SD. Differences between the means of 2 groups was analyzed by the Student's *t*-test or Mann–Whitney U-test using GraphPad Prism software (version 6.0c). Pearson's correlation analysis was also performed by GraphPad Prism software (version 6.0c). $P < 0.05$ was considered significant.

RESULTS

Consistency of CM assessment by neurological symptoms and BBB integrity

Neurological symptoms are the most representative manifestation of CM, including ataxia, paralysis, convulsions, etc. Therefore, an ECM clinical scoring system is commonly used to monitor the progress of the disease. The system counts ruffled fur, hunching, wobbly gait, limb paralysis, convulsions, and coma. Each symptom represents a score of 1, and if the total score ≥ 4, the mouse is diagnosed with CM. Based on these criteria, we classified the infected mice into NCM and CM.

Disruption of the BBB occurs during various central nervous system diseases. The permeability of the BBB can be evaluated by Evans Blue staining. In CM, the BBB is disrupted due to sequestration of pRBCs in the brain microvasculature. Therefore, we injected mice with Evans Blue to assess BBB integrity and confirm the CM diagnosis (Fig. 1). We evaluated 120 mice from day 5 to day 14 p.i. using these 2 methods (Table I). If a mouse presented with neurological symptoms and was diagnosed with CM by the clinical scoring system, it was injected with Evans Blue solution to check the permeability of the BBB. The ECM clinical scores and Evans Blue extravasation values of 120 mice were displayed in Suppl. File 1. Pearson's correlation analysis demonstrated that these 2 variables were significantly correlated ($P < 0.0001$, $R^2 = 0.6219$). Evans Blue staining revealed a false-positive rate (FPR) of 6.56% and false-negative rate (FNR) of 5.08% for the ECM clinical scoring system. These results indicate that the ECM clinical scoring system is reliable and practical in CM assessment.

ECM morbidity is inoculum dose-dependent and unstable

Inoculation of 1×10^4 to 1×10^6 pRBCs has been suggested for the induction of ECM. The administration route can be tail vein (IV) injection or intraperitoneal (IP) injection. To investigate whether an even lower dosage would be more effective, we

Table I. Consistency of cerebral malaria (CM) assessment by neurological symptoms and blood-brain barrier (BBB) integrity*.

Batch		BBB Leakage (+)	BBB Leakage (−)
1	CM symptoms (+)	7	0
	CM symptoms (−)	0	9
2	CM symptoms (+)	10	0
	CM symptoms (−)	0	2
3	CM symptoms (+)	8	1
	CM symptoms (−)	0	13
4	CM symptoms (+)	13	1
	CM symptoms (−)	0	0
5	CM symptoms (+)	12	0
	CM symptoms (−)	3	5
6	CM symptoms (+)	6	2
	CM symptoms (−)	0	28
Total	CM symptoms (+)	56	4
	CM symptoms (−)	3	57

* False positive rate = 6.56%; false negative rate = 5.08%.

compared the injection of 1×10^3 and 1×10^6 pRBCs. Based on a previous protocol for ECM construction (de Oca et al, 2013), we tested a murine model of CM in 7 independent batches of experiments, with an inoculum dose of 1×10^3 or 1×10^6 pRBCs intraperitoneally or intravenously. The general incidence of CM in the different batches of experiments varied from 13.51% to 93.33%, which is not as consistent as described previously (Rest, 1982). When 1×10^3 and 1×10^6 pRBCs were inoculated via the same method of injection in the same batch of experiments, inoculation with 10^3 pRBCs always had higher efficiency at establishing ECM than 10^6 pRBCs (Table II). All the mice with CM were confirmed by Evans Blue staining. The parasitemia of 6 batches of experiments are shown in Suppl. File 2. These results indicate that only some of the animals infected by the common inoculation dose will develop CM, and a lower dose will induce a relatively higher incidence of CM.

Differences in serum biochemical indices between NCM and CM

To gain insight into the differences between low and high inoculum doses to induce CM, we analyzed biochemical indices in sera collected from uninfected control mice (n = 5), CM mice (n = 7, 1×10^3 pRBCs; n = 11, 1×10^6 pRBCs), and NCM mice (n = 15, 1×10^3 pRBCs; n = 5, 1×10^6 pRBCs). The sera samples from CM mice were collected when the mice presented with neurological symptoms 6–8 days p.i. for the 10^6 pRBC inoculum group and 8–10 days p.i. for the 10^3 group. The sera samples from NCM mice were collected 8 days p.i. for the 10^6 group and 10 days p.i. for the 10^3 group. The uninfected control mice were sacrificed on day 10. In general, compared to the control group, ALT and AST, which represent liver function, increased significantly in both the NCM and CM groups, and liver damage was more severe in the CM ($P < 0.05$; Fig. 2a). BUN levels tended to be elevated in the CM group relative to the NCM and uninfected groups without a remarkable change, whereas the Cr level did not change (Fig. 2b). TC concentrations were enhanced in the sera of CM mice compared to the NCM mice ($P < 0.05$; Fig. 3a), whereas HDL-c and LDL-c levels were significantly decreased in CM sera compared to NCM ($P < 0.05$; Fig. 3b). Further analysis found that TC levels in the 10^3 pRBC-infected CM group were higher than in the 10^6 pRBC-infected CM group (Fig. 3a), whereas TGs

Table II. Percentage of mice with cerebral malaria (CM) symptoms.

Batch	Infective dose	Injection method*	Mice (n)	CM incidence (%)
1	10^6	i.p.	16	43.75
2	10^3	i.p.	13	84.62
3	10^3	i.p.	23	39.13
4	10^3	i.v.	15	93.33
5	10^3	i.p.	11	81.82
	10^6	i.p.	14	78.57
6	10^3	i.v.	20	40.00
	10^6	i.v.	22	18.18
7	10^3	i.p.	23	73.91
	10^6	i.p.	37	13.51

* i.p., interperitoneal; i.v., intravenous.

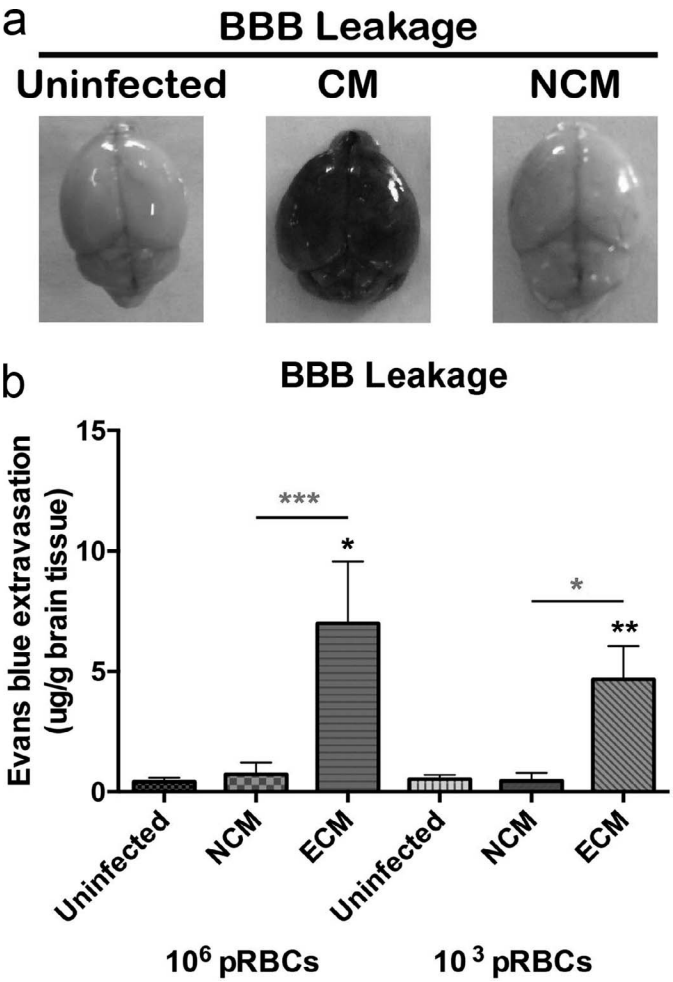

Figure 1. Assessment of blood-brain barrier (BBB) integrity in experimental cerebral malaria by Evans blue staining. (**a**) Representative brain images show BBB leakage using the Evans blue staining method. (**b**) Quantitative assessment of BBB leakage presented as the optical density of brain extracts at 630 nm. *$P < 0.05$; **$P < 0.01$, ***$P < 0.001$ (Mann–Whitney test). Error bars represent SD.

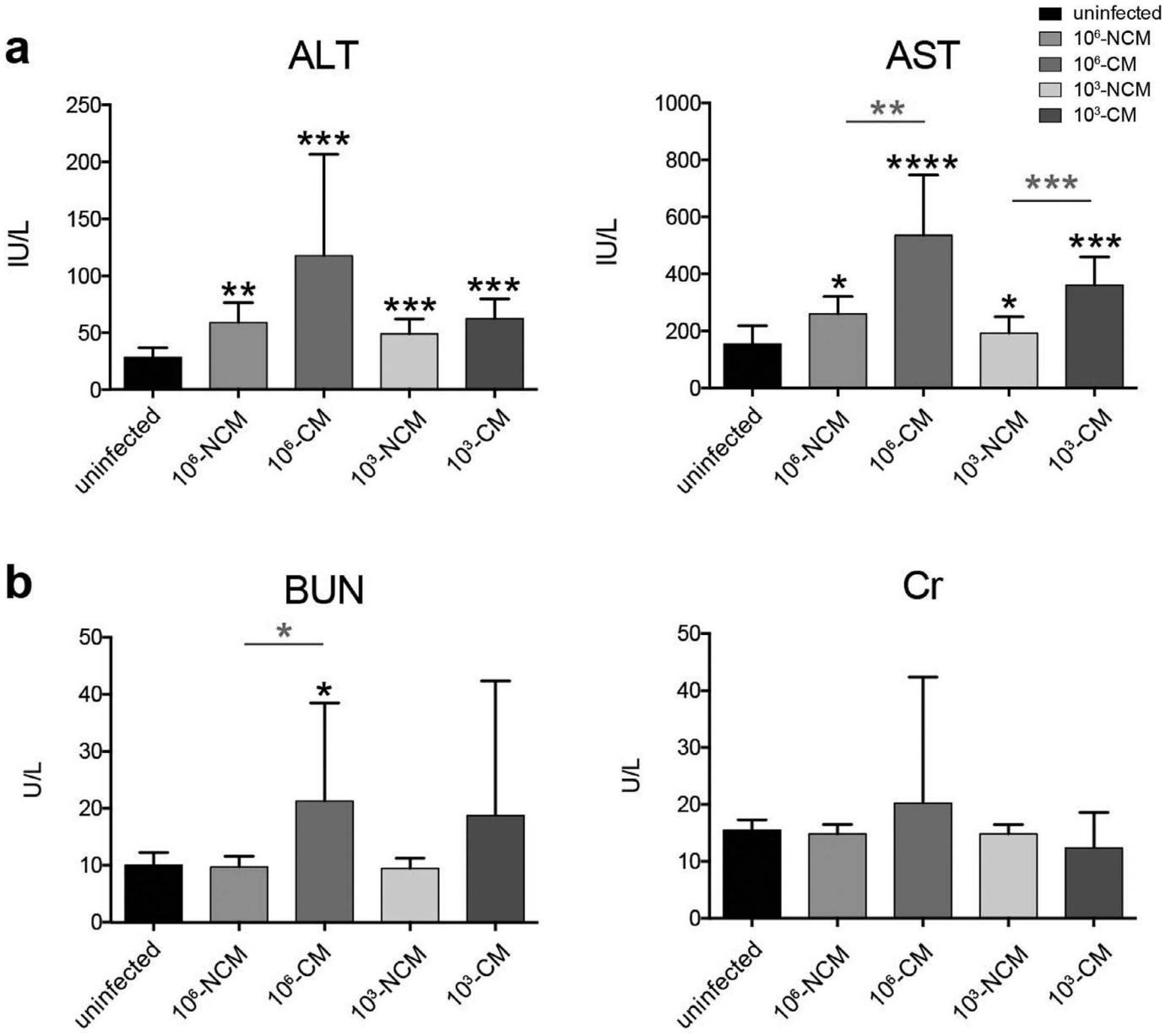

Figure 2. Impaired liver and kidney function in non-cerebral malaria (NCM) and cerebral malaria (CM). (**a**) Serum ALT and AST levels in uninfected mice, NCM and CM mice inoculated with 1×10^6 pRBCs, and NCM and CM mice inoculated with 1×10^3 pRBCs. (**b**) Serum BUN and Cr levels in the 5 groups described above. $^*P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ (Mann–Whitney test). Error bars represent SD. Abbreviations: ALT, alanine aminotransferase; AST, aspartate transaminase; BUN, blood urea nitrogen; CM, cerebral malaria; Cr, creatinine; NCM, non-cerebral malaria; pRBCs, parasitized red blood cells.

and VLDL-c levels were significantly elevated in the 10^3 pRBC-infected NCM group compared to the 10^6 pRBC-infected NCM group (Fig. 3). These results suggest characteristic blood lipid levels and liver dysfunction in ECM. In addition, different infection doses led to variations in blood lipid levels in NCM and CM mice, implying a different pathogenesis involved in ECM established by different initial infection doses.

DISCUSSION

Cerebral malaria is a neurological syndrome with a poor prognosis and an elusive mechanism. Although the murine ECM model cannot reproduce all of the features of HCM, it is still a powerful tool for studying CM pathogenesis (Strangward et al., 2017). However, the protocols for ECM construction vary without a unified standard. Some reports have declared a 100% incidence of CM (Rest, 1982), whereas other studies of ECM have not referred to the exact morbidity (Gramaglia et al., 2006; Crowley et al., 2017; Gun et al., 2017). The development of murine CM is dependent on the mouse strain (Rest, 1982; Curfs et al., 1992; Amani et al., 1998), the parasite strain (Engwerda et al., 2005), the clonal diversity of the parasite (Amani et al., 1998), the age of the mice (Hearn et al., 2000), and the dose of inoculated pRBCs (Curfs et al., 1992; Hein-Kristensen et al., 2010). Due to

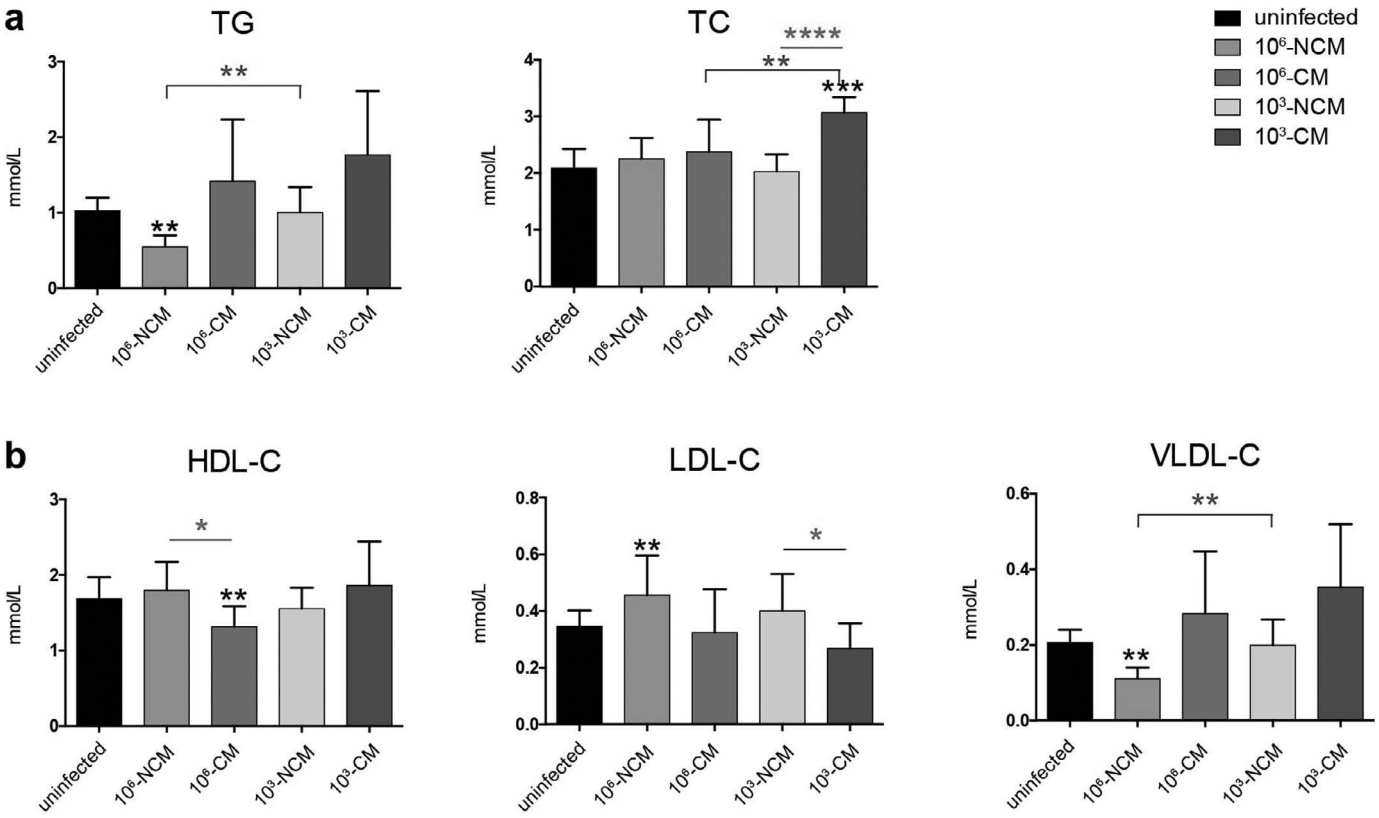

Figure 3. Blood lipid level alterations in experimental cerebral malaria (ECM) pathogenesis. (**a**) Serum TG and TC levels in uninfected mice, NCM and CM mice inoculated with 1×10^6 pRBCs, and NCM and CM mice inoculated with 1×10^3 pRBCs. (**b**) Serum HDL-c, LDL-c, and VLDL-c levels in the 5 groups described above. $^*P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ (Mann–Whitney test). Error bars represent SD. Abbreviations: CM, cerebral malaria; HDL-c, high-density lipoprotein-cholesterol; LDL-c, low-density lipoprotein-cholesterol; pRBCs, parasitized red blood cells; NCM, non-cerebral malaria; TC, total cholesterol; TG, total triglycerides; VLDL-c, very low-density lipoprotein-cholesterol.

variations in the induction of CM in different strains of mice with different strains of parasites, we chose the most frequently used C57BL/6 mice and *P. berghei* ANKA for this study. We found that the morbidity of ECM fluctuated among each experiment batch in correlation with the inoculum dose. The higher the inoculum dose, the lower the percentage of mice that developed CM. This phenomenon is consistent with several previous studies (Curfs et al., 1992; Hein-Kristensen et al., 2010). However, the incidence of CM still fluctuates greatly, even with the same low inoculum dose (e.g., 1×10^3 pRBCs) and the same injection method (IP) in distinct experiments, varying from 39% to 84% in the current study. While referring to the breakdown of BBB, there were no difference of Evan Blue's leakage into the brain tissues between low and high inoculum doses induced cerebral malaria. We have chosen experimental animals of the same strain and age and infected these mice with the same strain of parasite and dose. It is possible that the observed variation is related to subtle differences in parasitemia and the strain of initial passage mice. Alternatively, the precise weight and/or health conditions of the donor and acceptor animals play a role as well. It is impractical to synchronize these parameters prior infection. Therefore, careful verification of the ECM construction appears to be necessary.

A meta-analysis suggested specific lipid profiles in malaria patients compared to healthy controls (Visser et al., 2013), including lower TC, HDL-c, and LDL-c concentrations. Changes in the serum lipid profile also correlate with the disease progression in malaria patients (Mesquita et al., 2016). Another study of mice using nuclear magnetic resonance (NMR) detected an increased level of TGs and VLDL-c in CM compared to NCM (Ghosh et al., 2012). In our study, elevated TC and decreased HDL-c and LDL-c was found in CM compared to NCM. The increased TC levels had the same tendency discovered in the research described above (Ghosh et al., 2012). Serum cholesterol levels are considered to have a reverse trend with parasitemia in malaria patients, and CM always develops with relatively lower parasitemia. Thus, it is reasonable that the TC level is higher in CM than in NCM. TC levels were higher in the 10^3 pRBC-infected CM group than the 10^6 pRBC-infected CM group, and TGs and VLDL-c levels were higher in the 10^3 pRBC-infected NCM group than the 10^6 pRBC-infected NCM group. Host lipids play important roles in parasite proliferation, metabolism (Gulati et al., 2015), and hemozoin formation (Bendrat et al., 1995). These results indicate that different infection doses may induce different growth situations for parasites, leading to distinct lipid profiles. Furthermore, liver damage is also involved in CM pathogenesis. Lipids are synthesized in the liver, and liver damage is considered to occur in ECM prior to the onset of neurological symptoms (Haque et al., 2011), making it a potential early diagnostic marker.

In conclusion, we found that the induction of ECM according to the conventional protocol was unstable, and a lower inoculum dose may result in a higher incidence of murine CM. Experimen-

tal cerebral malaria induced by low dose inoculation still requires additional verification for efficiency. Biochemical analysis of ECM mice revealed characteristic serum lipid profiles and liver dysfunction. These findings provide new clues for the diagnosis and mechanistic probing of CM pathogenesis.

Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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