Establishment of a murine model of cerebral malaria in KunMing mice infected with *Plasmodium berghei* ANKA

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SUMMARY

Malaria remains one of the most devastating diseases. Cerebral malaria (CM) is a severe complication of *Plasmodium falciparum* infection resulting in high mortality and morbidity worldwide. Analysis of precise mechanisms of CM in humans is difficult for ethical reasons and animal models of CM have been employed to study malaria pathogenesis. Here, we describe a new experimental cerebral malaria (ECM) model with *Plasmodium berghei* ANKA infection in KunMing (KM) mice. KM mice developed ECM after blood-stage or sporozoites infection, and the development of ECM in KM mice has a dose-dependent relationship with sporozoites inoculums. Histopathological findings revealed important features associated with ECM, including accumulation of mononuclear cells and red blood cells in brain microvascular, and brain parenchymal haemorrhages. Blood–brain barrier (BBB) examination showed that BBB disruption was present in infected KM mice when displaying clinical signs of CM. *In vivo* bioluminescent imaging experiment indicated that parasitized red blood cells accumulated in most vital organs including heart, lung, spleen, kidney, liver and brain. The levels of inflammatory cytokines interferon-gamma, tumour necrosis factor-alpha, interleukin (IL)-17, IL-12, IL-6 and IL-10 were all remarkably increased in KM mice infected with *P. berghei* ANKA. This study indicates that *P. berghei* ANKA infection in KM mice can be used as ECM model to extend further research on genetic, pharmacological and vaccine studies of CM.

Key words: cerebral malaria, murine model, pathogenesis, immune response, *Plasmodium berghei* ANKA, KM mice.

INTRODUCTION

Malaria is a devastating disease that caused by parasitic protozoan of *Plasmodium*, leading to approximately 200 million infections and 600 000 deaths per year worldwide (WHO, 2014). Cerebral malaria (CM) is a severe complication of *P. falciparum* infection and is the main cause of death in patients with malaria (Murray *et al*. 2012). In most endemic areas of Africa, 1120 in 100 000 malaria patients develop CM every year, and high-risk groups is pre-school children, with 500 000 cases annually and mortality rate is 18·6% (Breman, 2001). Since the early clinical symptoms of CM are very similar to other diseases (Berkley *et al*. 1999), such as encephalitis, meningitis or febrile convulsions, optimal treatment opportunity tend to be miss or delay. Without in time treatment, CM can progress rapidly and patients might have died in 14 days after infection (Warrell *et al*. 1990). Even with efficacious intervention, mortality is still 15–22% (Idro *et al*. 2005). Moreover, for children who survived from CM, early studies suggested that they will make a full recovery (Muntendam *et al*. 1996), but recent accumulating evidence indicated that these survivors will suffer long-term cognitive impairment and behaviour problems (Idro *et al*. 2010*a*, *b*). Therefore, uncovering the mechanisms of pathogenesis of CM is a prerequisite for the formulation of effective prevention and treatment strategies.

Due to ethical constraints, studies on the mechanisms of CM rely on animal models, which include primate model [squirrel monkeys infected with *Plasmodium falciparum* (Gysin *et al*. 1992), rhesus monkeys infected with *P. knowlesi* or *P. coatneyi* (Ibiwoye *et al*. 1993)] and murine model. The former also has ethical issues and high cost of experiment is the major obstacle to its widely used. The latter has several combinations that could induce mice to appear a series of pathological features that are similar to CM. The almost exclusively used combination in recent years is *P. berghei* ANKA (PbA) infection in C57BL/6 or CBA mice (de Souza *et al*. 2010). The findings from this model demonstrated that CM is most likely the result of excessive pro-inflammatory immune response to the infection. Various immune cells and cytokines appear to be involved in the development of CM. Dendritic cells (de Walick *et al*. 2007; Lundie *et al*. 2008), natural killer cells (Hansen *et al*. 2007) are required for CM disease induction. Interferon-γ (IFN-γ) (Belnoue *et al*. 2008; Villegas-Mendez *et al*. 2011) and lymphotoxin-α (Engwerda *et al*. 2002) seems to play a pivotal role in driving the immunopathological process

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leading to CM. Chemokine receptor CXCR3 and its ligands CXCL9 and CXCL10 mediate monocytes and activated T-lymphocytes migration to the brain (Campanella *et al*. 2008; Miu *et al*. 2008). In particular, brain-accumulated cytotoxic $CD8^+$ T cells provoke CM pathogenesis via perforin- and granzyme B-dependent manner (Haque *et al*. 2011). In view of the complexity of CM, it is likely that host genetic makeup is related to the ultimate outcome of the disease (Lovegrove *et al*. 2006). Several genes or loci were found to play a pivotal role in determining the susceptibility of host to infection (Bagot *et al*. 2002). Although inbred mouse model exhibit a number of advantages in CM studies, the application of outbred mouse model can show potential usefulness in the identification of genes or regulators influencing susceptibility or resistance to CM, as well as antimalarial drug and vaccine studies (Martins *et al*. 2009). Thus, developing new outbred mouse model with a combination of host and parasite able to appropriately mimic the disease seen in humans is helpful.

In this study, we developed a new experimental cerebral malaria (ECM) model through the using of KunMing (KM) mice. This outbred mouse line stems from Indian Haffkine Institute in 1944 and exhibits high disease resistance, good adaptive capacity, high breeding coefficient and good survival rate. Now it has been widely applied in pharmacological, toxicological, medicinal and biological research and testing in China (Shang *et al*. 2009). PbA infection in KM mice can reproduce many neurological signs and pathological features of human CM. By using this model, we may be able to extend further research on discovering the genes or regulators determining susceptibility or resistance to CM and evaluate strategies for the development of vaccines and drugs.

MATERIALS AND METHODS

Mice and ethics statement

Specific-pathogen-free female KM mice (6–8 weeks of age) were purchased from the Laboratory Animal Center of Third Military Medical University (Chongqing, China). Animal care and all experiments were conducted in accordance with the approved protocols by the Institutional Animal Care and Use Committee of the Third Military Medical University.

Mosquito rearing and infection

Anopheles stephensi (Hor strain) were routinely fed and infected in our laboratory. Briefly, mosquitoes were maintained with a 5% sugar solution at a relative humidity of 70–80%. For infection with PbA, 3- to 5-day-old female adults were fed on the PbA-infected KM mice and were maintained at 19–21 °C post-blood feeding. At 20 days post infection (dpi), sporozoites were collected from infected mosquitoes.

Parasites, infections and disease evaluation

PbA parasite and a transgenic PbA parasite (231c1 l) expressing luciferase under the control of the ef1a promoter (PbA-luc) (Amante *et al*. 2007) and *P. berghei* NK65 (PbNK) were maintained in our laboratory by passage through naive mice. Experimental infections were initiated by intraperitoneal inoculation of 10^6 parasitized red blood cells (pRBCs) or intravenous inoculation of 1×10^3, 5×10^3, 1×10^4, 5×10^4 sporozoites. Parasitaemia was determined by examination of Giemsa-stained thin blood smears on different days post infection. Infected mice were monitored daily for neurologic signs of ECM such as ataxia, paralysis, convulsion or coma, animals that showed these signs and died within 12 dpi were considered as having ECM (Amani *et al*. 1998) and cumulative ECM incidence was then reported.

Blood–brain barrier (BBB) studies

Mice were intravenous injection of 200 μL of 2% Evans blue dye (Sigma). One hour later, the brains were removed and placed in formamide for 48 h after intracardial perfusion with phosphate-buffered saline (PBS), and dye extravasation was determined as previously described (van der Heyde *et al*. 2001). Absorbance of dye was measured at 620 nm (Bio-Rad). Using a standard curve, data were calculated and expressed as μg Evans blue dye/g brain tissue.

Histopathological examination

The brains were fixed in 4% paraformaldehyde, paraffin-embedded, sectioned (5 μm), and stained with haematoxylin and eosin (H&E). Non-infected KM mice and PbNK-infected KM mice were used as controls and sacrificed at the same time point. For histological analysis, brain sections were examined in a blind fashion.

Cytokine detection

Blood samples were collected from PbA-infected KM mice and PbNK-infected KM mice at 2, 4, 6 dpi. The levels of pro-inflammatory cytokines IFN-γ, tumour necrosis factor-alpha (TNF-α), interleukin (IL)-17, IL-12, IL-6 and anti-inflammatory cytokine IL-10 in plasma were determined by flow cytometry using the CBA mouse inflammation kit (BD, USA) according to the manufacturer's protocol.

In vivo bioluminescent imaging

Parasites distribution in KM mice infected with PbA-luc was assessed by an *in vivo* imaging system (IVIS Spectrum, Xenogen, Alameda, CA) as

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reported previously (Franke-Fayard *et al*. 2006). When ECM symptoms were observed, PbA–luc-infected KM mice were anesthetized with fluorothane and injected intraperitoneally (i.p.) with 0·1 mL of 5 mg mL^{-1} D-luciferin (Xenogen). 5 min later, Whole body bioluminescence imaging was captured on the IVIS according to the manufacturer's instructions. For organs bioluminescence imaging, PbA–luc-infected KM mice were received a second i.p. injection of luciferin, and organs were dissected after intracardial perfusion. Bioluminescence was measured as photons/second/cm^2/steer radiant (p s^{-1} cm^{-2} sr^{-1}) using Living Image software (Xenogen).

Real-time PCR analysis

Mice that displayed clinical symptoms of ECM were sacrificed and perfused intracardially with PBS to remove non-adherent lymphocytes. Then brains were homogenized in TRIzol Reagent (Invitrogen) and total RNA was isolated according to the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA in a 10 μL reaction using Moloney Murine Leukemia Virus Reverse Transcriptase (Promega) with oligo dT primers (Invitrogen). The brain-sequestered $CD8^+$ T cells were evaluated by detecting the relative expression of CD8β gene using the SYBR Premix EX Taq (Takara) on Eco Real-Time PCR system (Illumina, San Diego, CA). Housekeeping gene *hprt* was used as an endogenous reference and data were shown relative to a control sample after *hprt* normalization. Mouse *hprt* and CD8β-specific primer sequences were 5′–TGCTCGAGATGTGATGAAGG–3′ and 5′–TCCCCTGTTGACTGGTCATT–3′; and 5′–TGCTCGAGATGTGATGAAGG–3′ and 5′–TCCCCTGTTGACTGGTCATT–3′, respectively.

Statistical analysis

Statistical significance was determined with GraphPad Prism Version 6.0 (GraphPad Software Inc., La Jolla, CA). Dates are expressed as mean ± SD indicated by error bars unless indicated otherwise. Differences were evaluated by non-parametric Mann–Whitney *U* test or Log-rank test for survival. $P < 0{\cdot}05$ was considered significant.

RESULTS

KM mice develop ECM after blood-stage infection

To investigate whether KM mice infected with PbA would develop ECM, we first used pRBCs to initiate infection and employed PbNK-infected KM mice as a control. It has been reported that PbNK was a non-ECM-causing parasite line. After injection of 10^6 pRBCs, parasitemia levels in PbA-infected KM mice increased rapidly from 2 dpi and peak parasitaemia was observed at 6 dpi, whereas PbNK-infected KM mice had moderate parasitaemia at 6 dpi (Fig. 1A). The manifestations of CM in mice were characterized by clinical signs such as ataxia, paralysis, disorientation, convulsions and coma. These neurological signs were commonly observed in the PbA-infected KM mice. These mice suffering from ECM usually died within a short time (<24 h). All PbA-infected KM mice succumbed to ECM until 9 dpi. In contrast, PbNK-infected KM mice did not show signs of ECM at the same time and died about 24 dpi with high parasitaemia (Fig. 1A–C). Significant weight loss and red blood cells decrease were also observed in KM mice infected with PbA in comparison with PbNK-infected mice (Fig. 1D and E).

Sporozoites infection induces ECM in KM mice via dose-dependent manner

Since i.p. injection of asexual blood-stage parasites bypasses the obligate liver-stage phases of the life cycle and how the initial liver stages may shape responses to blood-stage parasites is largely unknown, the results from blood-stage PbA infection need to be confirmed. Thus, we infected KM mice with different doses of sporozoites (1×10^3, 5×10^3, 1×10^4, 5×10^4). We observed that the proportion of mice that developed ECM was dependent on the number of sporozoites used to initiate infection, with higher quantities leading to increased incidence of ECM (Fig. 2A). Given that sporozoites have to complete the obligate liver stage before entering the blood stage, ECM symptoms supposed to appear later in sporozoites-infected mice than in blood-stage-infected mice. Surprisingly, the clinical signs of ECM appeared earlier in sporozoites-infected mice than in pRBCs-infected mice, even though the former had a significantly lower parasitaemia than the latter between 4 and 6 dpi (Fig. 2B and C). No significant difference in parasitaemia levels was seen between the groups of mice infected with different sporozoites in the period of infection (Fig. 2B).

KM mice show severe alterations in the brain after PbA infection

As BBB disruption is a hallmark of ECM as well as of CM in humans (Adams *et al*. 2002), we next examined the brains of PbA-infected mice and those of PbNK-infected mice at 6 dpi after injection of 10^6 pRBCs (when PbA-infected mice displayed typical symptoms of ECM). In KM mice, infection with PbNK did not lead to BBB disruption. In contrast, PbA-infected mice showed BBB disruption, as revealed by abundant dye leakage in the brain (Fig. 3A). Accumulated Evans blue in brain parenchyma of PbA-infected mice was significantly higher as compared with PbNK-infected mice

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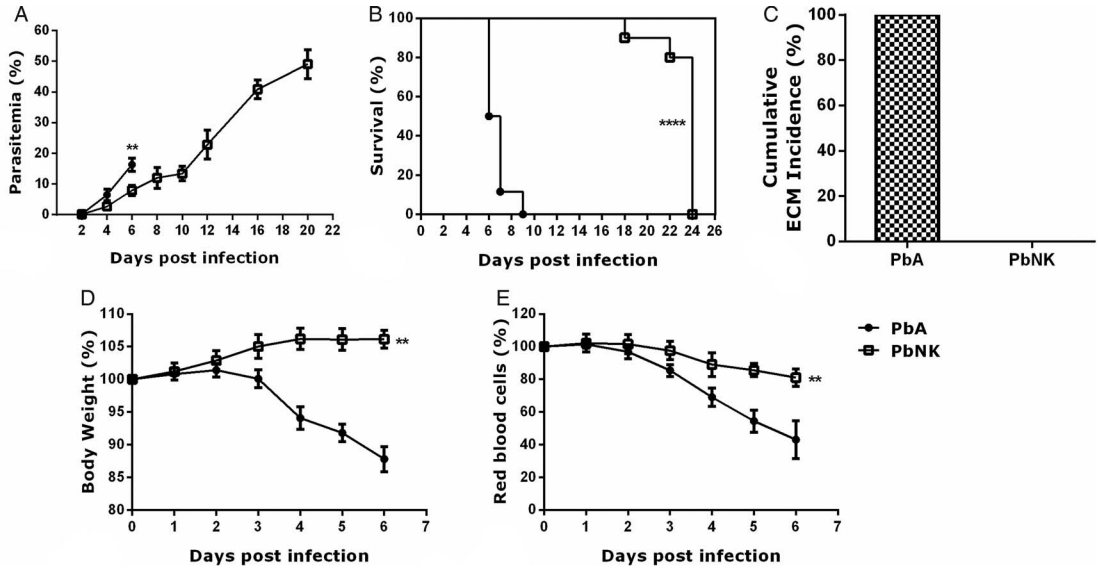

Fig. 1. Course of PbA and PbNK infection in KM mice. KM mice were injected i.p. with 10^6 PbA or PbNK parasites. Parasitaemia (A), survival (B), cumulative ECM incidence (C), body weight (D) and red blood cells (E) were recorded at selected time point. $n = 25$ mice per group. Parasitaemia, body weight and red blood cells were evaluated by non-parametric Mann–Whitney U test at 6 dpi and Log-rank test was used for survival. $^{**}P < 0{\cdot}01$; $^{****}P < 0{\cdot}0001$. The data are representatives of three independent experiments.

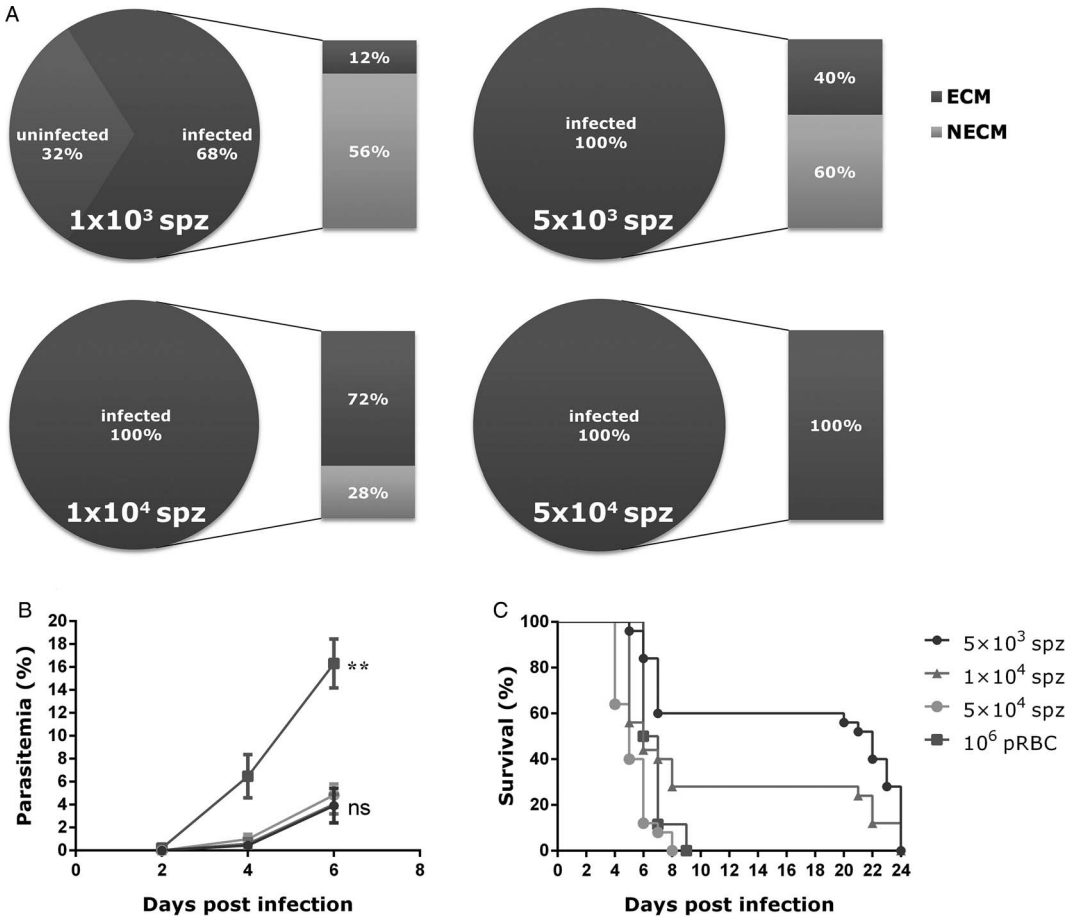

Fig. 2. Comparison of ECM in KM mice infected with different doses of sporozoites. (A) ECM incidence were evaluated in KM mice infected with different doses of sporozoites (1×10^3, 5×10^3, 1×10^4, 5×10^4). Parasitaemia (B) and survival (C) were assessed during the course of infection for mice infected with sporozoites or 10^6 pRBCs. $n = 25$ mice per group. The data are representatives of two independent experiments. $^{**}P < 0{\cdot}01$; ns, non-significant, non-parametric Mann–Whitney U test. NECM, no experimental cerebral malaria; spz, sporozoites.

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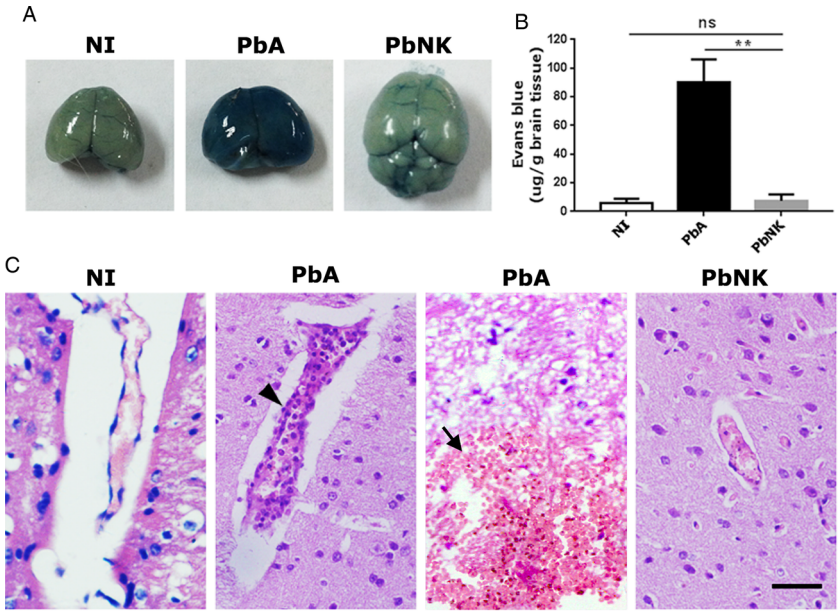

Fig. 3. Severe alterations in the brain of KM mice after PbA infection. BBB disruption was assessed in PbA-infected mice, PbNK-infected KM mice or non-infected (NI) mice by Evans blue at 6 dpi (A, B). Histopathological evaluation of the brain was assessed by H&E staining at same time (C). $n = 5$ mice per group. $^{**}P < 0{\cdot}01$; ns, non-significant, Kruskal–Wallis test. Arrowhead show brain microvascular congestion and arrow show brain haemorrhage in PbA-infected mice. Bar = 100 μm.

(Fig. 3B). We also performed histological examination of brain tissue at 6 dpi, and results were consistent with BBB examination. PbA-infected mice exhibited pathological features associated with ECM, including accumulation of mononuclear cells and brain parenchymal haemorrhages. These features were not detectable in KM mice infected with PbNK (Fig. 3C).

KM mice show parasite burden in the brain and exhibit exaggerated pro-inflammatory response after PbA infection

It is well established that human CM is correlated with extensive pRBCs sequestration in brain microvasculature, so we questioned, whether ECM KM mice will reproduce this important phenomenon. To that end, KM mice were infected with a transgenic PbA strain that constitutively expresses luciferase (Amante *et al*. 2007). By using PbA-luc line, we were able to evaluate parasites that had sequestered in tissues. On 6 dpi, when KM mice were displaying symptoms of ECM, parasite-derived bioluminescence were measured. We observed great parasite biomass in the whole body of KM mice and some vital organs, such as brain, lung and heart, exhibited high parasite burden and highest burden was seen in the lungs (Fig. 4A). Next, we carried out a more detailed analysis of the sequestration of pRBCs in KM mice. After a second luciferin injection followed by intracardiac perfusion to remove circulating blood, various organs were dissected for bioluminescence imaging. High parasite burden appeared in most vital organs including heart, lung, spleen, kidney, liver and brain (Fig. 4B and C). These data demonstrate that pRBCs accumulated in vital organs of KM mice when they displaying typical signs of ECM after PbA infection. In addition, we assessed whether $CD8^+$ T cells were sequestered in the brain, another primary effector in the end-stage of ECM (Haque *et al*. 2011). There was no difference in CD8β mRNA expression in the brains at 6 dpi between PbNK-infected mice and PbA-infected mice (Fig. 4D).

It was reported that the levels of cytokines have important roles in the development of CM. We next evaluated the inflammatory response of KM mice after PbA and PbNK infection. The pro-inflammatory cytokines IFN-γ, TNF-α, IL-17, IL-12 and IL-6 and the anti-inflammatory cytokine IL-10 were measured at 2, 4, 6 dpi. The pro-inflammatory cytokines IFN-γ, TNF-α, IL-17, IL-12 and IL-6 began to increase after infection and were significantly elevated in mice infected with PbA, while these cytokines increased mildly in mice infected with PbNK (Fig. 5). The anti-inflammatory cytokine IL-10 also significantly increased in PbA-infected mice and a relatively slow increase in PbNK-infected mice during the course of infection. The levels of all cytokines in PbA-infected mice were significantly higher than in PbNK-infected mice (Fig. 5).

DISCUSSION

CM is a lethal complication of *P. falciparum* infection in children under five, and the picture to date

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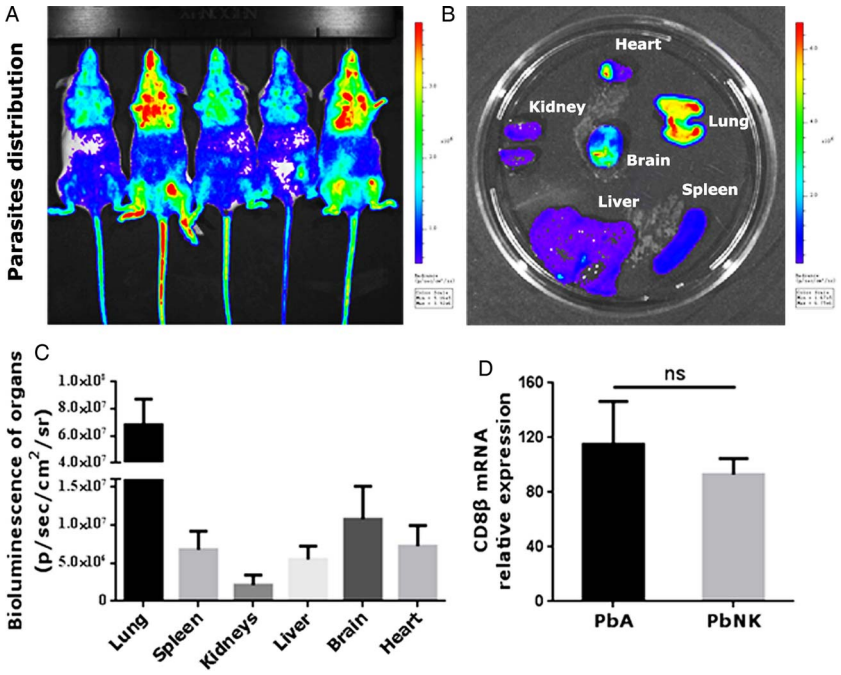

Fig. 4. Parasite burden in PbA–luc-infected KM mice. All KM mice were infected with 10^6 PbA–luc pRBCs. On 6 dpi, when mice were exhibiting typical signs of ECM, *in vivo* bioluminescence imaging of pRBCs accumulation in the whole body (A) and dissected organs (B) of PbA–luc-infected KM mice was performed after luciferin injection and quantification of parasite burden in various organs (C) was determined after a second luciferin injection followed by intracardiac perfusion. Quantification of brain CD8β mRNA expression (D) was assessed by qRT–PCR. ns, non-significant, non-parametric Mann–Whitney *U* test. Data are representatives of two independent experiments.

of precise mechanisms of disease is still incomplete. Experimental animal models are valuable tool for unveiling underlying basis of CM (de Souza *et al*. 2010). The data presented in this study demonstrate for the first time that PbA infection in KM mice can reproduce many neurological signs and pathological features of human CM, and thus can be used as a new ECM model. Our findings show, firstly, that KM mice infected with PbA, not PbNK, can develop ECM and the development of ECM in KM mice has a dose-dependent relationship with sporozoites inoculums. Second, BBB disruption and pathological features are clearly evident in the brains of KM mice after PbA infection, while PbNK-infected mice maintain BBB integrity and pathological damages are absent from infection. Third, in agreement with the histopathological examination, KM mice display high parasite burden in the brains when showing typical symptoms of ECM. At last, the pro-inflammatory cytokines IFN-γ, TNF-α, IL-17, IL-12 and IL-6 were significantly elevated in KM mice after PbA infection.

After blood-stage infection, all KM mice developed ECM. ECM mice clearly manifested neurological signs of CM, such as paralysis, ataxia, convulsions, retinopathy and coma, and died within several hours after the onset of ECM. However, this approach tends to ignore a potential issue that i.p. or intravenous inoculation of asexual blood-stage parasites bypasses the obligate liver-stage phases of the life cycle, and how the initial liver stages may shape responses to blood-stage parasites remains elusive. The role of liver-stage on influencing subsequent immune responses to blood-stage is rarely studied (Bagot *et al*. 2004; Fonseca *et al*. 2007). In this study, we reexamined the outcome of PbA infection in KM mice by using sporozoites to initiate infection. We found that the accumulative incidence rate of ECM in KM mice was dependent on the inoculation number of sporozoites. For instance, sporozoites consistently induced ECM in a high proportion of mice with a minimum of 1×10^4. This finding is consistent with a previous report that the amount of PbA inoculum used affects the development of ECM in C57BL/6 mice (Amani *et al*. 1998). Furthermore, an interesting result emerged from this part. The clinical signs of ECM were supposed to appear later in mice infected with sporozoites than in pRBCs-infected ECM mice, because liver stage of rodent malaria parasite PbA undergoes about 44 h before the development of the blood stage (Khan *et al*. 1992). However, the outcome is just the opposite: ECM was first observed in mice inoculated with sporozoites and disease progression was roughly the same in sporozoites- and pRBCs-infected mice. Moreover, parasitaemia was significantly higher in mice infected with pRBC than in mice infected with sporozoites between 4 and 6 dpi. It is worth

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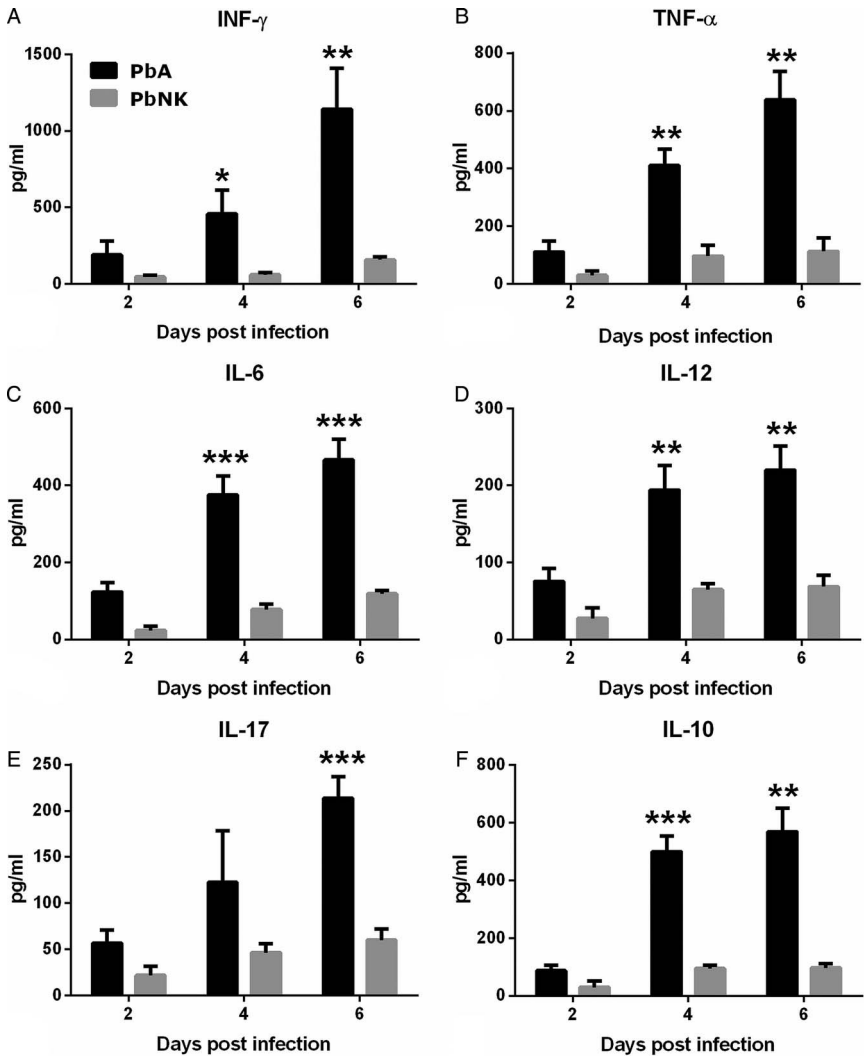

Fig. 5. Cytokines production of PbA-infected and PbNK-infected KM mice. KM mice were infected with 10^6 PbA or PbNK pRBCs. Blood samples were collected from these infected KM mice at 2, 4, 6 dpi. The levels of pro-inflammatory cytokines IFN-γ, TNF-α, IL-17, IL-12, IL-6 and anti-inflammatory cytokine IL-10 in plasma were determined by flow cytometry using the CBA mouse inflammation kit. *$P < 0{\cdot}05$; **$P < 0{\cdot}01$; ***$P < 0{\cdot}001$, non-parametric Mann–Whitney *U* test, all are PbA *vs* PbNK. Data are representatives of three independent experiments.

noting that different doses of sporozoites used did not have a direct relationship with the parasitaemia levels. Since sporozoites-infected mice contained significantly less parasites than that of blood-stage-infected mice when ECM first appeared, the results suggest that sporozoites are more efficient in priming the host immune system in comparison with pBRCs. In addition, these findings also indicate that the number of parasites used to initiate infection, but not circulating parasite in the blood (blood parasitaemia), play a critical role for the induction of ECM. At last, how liver-stage affects immune responses to blood-stage requires further investigations.

Sequestration of pRBCs and $CD8^+$ T cells in the brain are thought to be the two key events in the pathogenesis of ECM. It was suggested that accumulation of pRBC in the brain microvascular is the major histopathological feature of human CM and results in obstruction of vascular leading to cerebral hypoxia, haemorrhages, coma and death (Haldar *et al*. 2007). The data obtained from *in vivo* imaging experiment demonstrated that pRBCs sequestered in the brain of PbA-infected KM mice when showing typical signs of ECM. Mononuclear cells in brain microvascular, brain parenchymal haemorrhages and BBB disruption were also observed in PbA-infected KM mice, while these features were not detectable in KM mice infected with PbNK. Since peripheral blood parasitaemia only indicates circulating parasites, not tissue-sequestered parasites, we next used a transgenic PbA strain to explore parasites burden and tissue-sequestered parasites in KM mice after infection. The results showed that PbA–luc-infected KM mice displayed great parasite biomass in the whole body and brain harboured substantial parasites at 6 dpi when mice developed ECM.

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The role of $CD8^+$ T cells in the pathogenesis of ECM has also been established in a large number of experimental and clinical studies. It has previously been reported that $CD8\alpha^+$ dendritic cells are required for the activation of malaria-specific $CD8^+$ T cells in the spleen (Lundie *et al*. 2008) and primed $CD8^+$ T cells migrate to the brain with the help of chemokine receptor CXCR3 and its ligands CXCL9 and CXCL10 (Campanella *et al*. 2008; Miu *et al*. 2008). Recent research showed that activated $CD8^+$ T cells, in turn, aggravate accumulation of pRBCs in vital organs, including the brain (Amante *et al*. 2010; Claser *et al*. 2011). Finally, brain accumulated $CD8^+$ T cells exert a pathological effect through perforin- and granzyme-B-dependent mechanisms (Haque *et al*. 2011; Nitcheu *et al*. 2003). Furthermore, depletion of these cells before the appearance of neurological signs confers complete protection to mice against ECM, showing an unambiguous role for $CD8^+$ T cells in the end-stage of cerebral pathology (Belnoue *et al*. 2002; Renia *et al*. 2006). In this paper, we found that the difference in CD8b mRNA expression was insignificant between PbA infection and the non-ECM PbNK infection. Indeed, one recent study has shown that concurrent accumulation of pRBCs and $CD8^+$ T cells in the brain of C57/BL6 mice infected with PbA is crucial for the development of ECM (McQuillan *et al*. 2011). Brain sequestered $CD8^+$ T cells seem to be reactivated by accumulated pRBCs, which make brain microvascular endothelial cells a target for the immune response through transfer of parasite antigens to endothelial cells (Jambou *et al*. 2010). Exaggerated pro-inflammatory response has been shown to be closely related to the development of ECM during malaria infection. In our ECM model, all the pro-inflammatory cytokines, including IFN-γ, TNF-α, IL-17, IL-12 and IL-6 were found to be significantly increased in KM mice after PbA infection, consistent with the findings from murine inbred ECM models (Engwerda, 2005). Anti-inflammatory cytokine IL-10 is thought to exert immunosuppressive role in malaria infection. In our study, PbA infection also resulted in significantly increased levels of IL-10 in KM mice, but these mice eventually succumbed to ECM. This result suggested that one or a few immune regulators are inadequate to establish an appropriate balance between pro- and anti-inflammatory responses, which is crucial for the development of CM during the course of PbA infection.

The presence of resistant or susceptible mouse strains to CM indicated that host genetic background plays an essential role in determining the outcome of this deadly complication of malaria. Several genes or loci were found to play a pivotal role in determining the susceptibility of host to infection (Bagot *et al*. 2002). In this paper, results presented here provide solid evidences that KM mice developed ECM after PbA infection. Although inbred mouse model exhibit a number of advantages in CM studies, the application of outbred mouse model can show potential usefulness in a number of studies. Quantitative trait loci (QTL) research is one of the main applications of an outbred model for CM. A previous report showed that using outbred mice strain together with haplotype reconstruction to dissect a known quantitative trait locus influencing anxiety (Yalcin *et al*. 2004). This indicated the great potential of outbred mouse model for the exploration of genes linked to particular phenotypes (Chia *et al*. 2005). Another potential application for this outbred model of ECM is pharmacological and vaccine studies. KM mice have been widely applied in pharmacological, toxicological, medicinal and biological research and testing in China. At last, this outbred model could be a beneficial supplement to a number of existing murine models of CM in advancing our understanding of underlying mechanisms of CM.

In summary, we describe a new ECM model with PbA infection in KM mice. KM mice develop ECM after blood-stage or sporozoites infection and reproduce many neurological signs and pathological features of ECM. Moreover, our findings, based on this novel model, strongly support the view that pRBC and $CD8^+$ T cells are key players for the development of ECM.

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