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ORIGINAL PAPER

Histopathological studies in two strains of semi-immune mice infected with *Plasmodium berghei* ANKA after chronic exposure

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Abstract To mimic a human malaria infection in the endemic condition, two strains of mice (Balb/c and CBA) were infected and treated several times to generate so-called semi-immune status. As previously reported, neither mice (Balb/c and CBA) strain showed cerebral malaria, even in the susceptible C57BL/6 (B6). The significant difference between the mice strains in our previous study was the rate of destruction of uninfected red blood cells (uRBCs) at infection. After the established repeated cycles of infection and treatment and the final challenge with 10^4 *Plasmodium berghei* ANKA until minimum Hb, Balb/c and CBA mice were sacrificed. The spleen, liver, brain, kidney, lung, heart, and muscle were removed, stained with hematoxylin–eosin and analyzed with light microscopy. Previous observation suggested that Balb/c destroyed uRBC at much higher rate than the other strains although the parasitemia was very low. Pathological investigation carried out in this study revealed that this destruction was mainly contributed by the uRBCs as no parasite sequestration was observed in any of the organs. However, malaria pigment deposition was observed in spleen and liver of all the semi-immune mice strains. This histopathological study in the severe malaria anemia model, which is difficult to conduct in humans, will be helpful in taking into account different responses to malaria infection when designing therapeutic interventions and vaccine studies.

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Background

Immunity plays a critical role in helping individuals fight infection. Thus, immune responses to blood-stage malaria infection are in general deficient, with the need for long-term exposure to the parasite to achieve immunity. This results in the development of immunopathological states in some individuals such as cerebral malaria (CM) and severe malaria anemia (SMA) in most cases. However, with increase in age, the frequency of clinical attacks reduces, and after puberty most individuals except for pregnant women present some amount of immunity against clinical stages of malaria (Baird 1998; Druilhe and Perignon 1997). In the holo-endemic area in Ghana, for example, the major

cause of malaria death is severe anemia, whereas it is CM in the meso-endemic area. The reason why such difference occurred is not clear; however, it is highly possible that the frequent infection can enhance the destruction of RBC, during infection. This motivated us and others to use a semi-immune malaria model mouse (Evans et al. 2006; Helegbe et al. 2009) for the analysis of the pathogenesis of severe malaria anemia.

Hb loss in the semi-immune mice occurred at relatively low parasitemia and was speculated to be generated by the destruction of normal uninfected red blood cell (uRBC; Evans et al. 2006; Helegbe et al. 2009). Meanwhile, some studies observed that parasitemia level may confound anemia and thus actual parasite numbers may be related to anemia (Amante et al. 2007; Lamb and Langhorne 2008), suggesting that some parasites may be sequestered in some organs and not available in the peripheral blood as determined by percent parasitemia. Histopathological study on these organs will therefore be helpful to elucidate if these organs do sequester iRBC in the SMA model of the semi-immune individual. In most reports, histopathological studies have been carried in naïve animal models (Adun et al. 1965) and recently by Martins et al. (2009). However, not much characterization has been carried out in the SMA model of semi-immune mice strains.

The main advantage of the rodent model of SMA as developed by Evans et al. (2006) and used successfully in another study (Helegbe et al. 2009) is that they are uncomplicated by excessive parasite burdens, which is reflective of the associated hemolytic anemia of SMA in the human populations. Thus, with the variation in rates of RBC destruction observed in the different semi-immune mice strains and for the fact that actual parasite numbers have been implicated in the development of anemia (Lamb and Langhorne 2008), the histopathological studies of major organs responsible for RBC circulation, storage, and clearance has been carried out in two semi-immune mice strains in this study.

Methods

Mice, parasites, and infection

With our previous studies of high Hb loss at low parasitemia and high antibody titer in Balb/c, and low antibody titer with low Hb loss at relatively high parasitemia in CBA (Helegbe et al. 2009), we chose these two strains for the histopathology study to help understand further the mechanism of uRBC destruction in Balb/c. Thus, the two strains of mice Balb/c and CBA, aged 8 weeks supplied by SLC laboratories, Fukuoka, Japan, were injected intraperitoneally with 10^4 *Plasmodium berghei* ANKA (*Pb*ANKA)-infected RBCs. Parasitemia and reticulocyte levels were monitored every 2 days by Giemsa-stained thin blood film and are expressed as a percentage of more than 500 RBCs. Hemoglobin (Hb) was measured in a 96-well plate at 570 nm on Bio-Rad Model 3550 Micro plate Reader as previously described (Lamb and Langhorne 2008). Four microliter of tail-vein blood was suspended in 1 mL Drabkin reagent (Sigma, St Louis, MO) and absorbance was measured and expressed as a percentage of baseline levels. Laboratory and animal practices of the Animal Center of Institute of Tropical Medicine (NEKKEN), Nagasaki were adhered to after the approval from the local ethics committee for animal care and research was obtained.

Generation of semi-immune status in the mice strains

This was a modified method as described elsewhere (Evans et al. 2006). Two strains (Balb/c and CBA) of mice infected with 10^4 *P. berghei* ANKA, were treated at day 6 after infection with chloroquine (10 mg/kg intraperitoneally) and pyrimethamine (10 mg/kg intraperitoneally) daily for 6 days. During subsequent rounds of infection, mice were rested for 2 weeks before being rechallenged with 10^4 *P. berghei* ANKA, then monitored and drug cured prior to parasitemias reaching 5%. To evaluate if mice will be completely immunized, they underwent 14 cycles of drug-cured infection before finally being challenged with 10^4 *P. berghei* parasites without treatment. After the final cycle of infection, mice were monitored every other day and on days in which minimum Hb level (Fig. 1) was observed at parasitemia not exceeding 20%; mice were sacrificed and organs taken for histopathological study. Parasitemias exceeding 20% have been shown to complicate malaria anemia (Langhorne et al. 2002) and were excluded from analyses.

Histopathological study

The spleen, brain, liver, kidney, lung, heart, and muscle were fixed in 10% formalin until ready to be used. After fixation, the spleen, liver, and brain were cut in transversal sections, while kidney, lung, heart, and muscle were cut in longitudinal sections. These specimens were suspended in absolute alcohol, absolute xylene for 4 days, and embedded in paraffin. Sections were cut at 3.5 μm, stained with hematoxylin–eosin (HE) and analyzed by light microscopy. Same organs were obtained from uninfected mice and taken through the HE staining to serve as control. Results of histological findings were confirmed by at least an additional person. To further ensure the validity of the results, not less than ten fields per organ with similar microscopic field were observed carefully for histopatho-

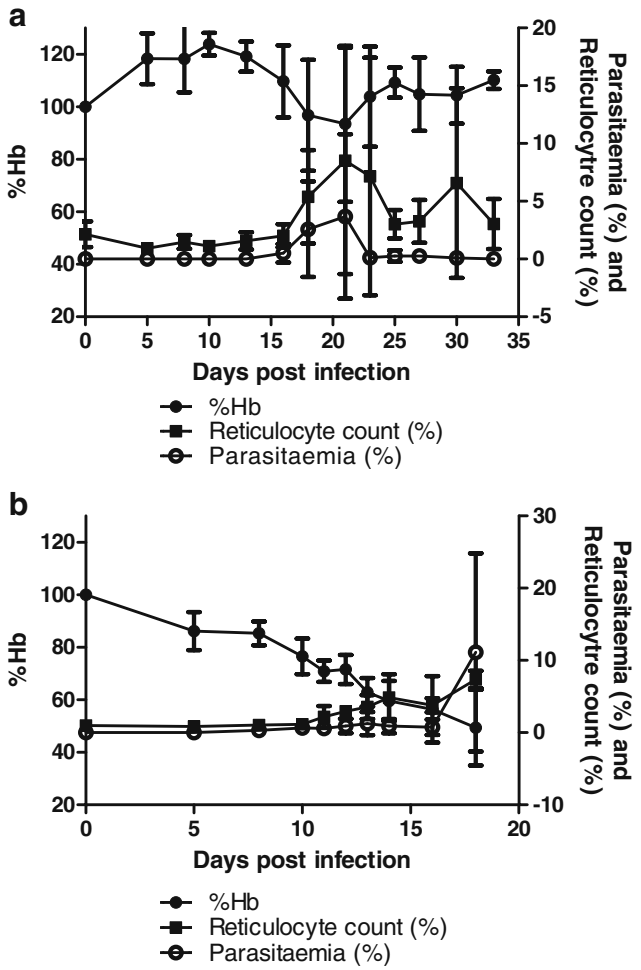

Fig. 1 Profile of malaria anemia in the semi-immune mice. Mean parasitemias, reticulocyte levels, and Hb in semi-immune mice strains **a** Balb/c, $n=6$, and **b** CBA, $n=7$, after *P. berghei* ANKA infection during the final cycle. Each mouse showed minimum %Hb level (maximal reduction) on different days. These are data from one experiment, and data are represented as mean±SD

logical signs of malaria pigment and sequestered iRBCs. Semi-qualitative approach was used to analyze and compare the variation in malaria pigment deposition among the semi-immune mice strains. For each histological section per animal not less than ten microscopic fields at 400× were randomly selected and examined for any histopathological changes, such as malaria pigment deposition and sequestered iRBCs.

Statistical analysis

Data analysis was done using the GraphPad Prism Version 5.00 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com. Data are expressed as the mean unless otherwise stated. Data were log transformed to ensure normal distribution before *t* test analysis was performed. Values were considered significant when $p<0.05$.

Results

Profile of parasitemia and hematological parameters in the semi-immune mice strains

Upon challenge with 10^4 *P. berghei* ANKA after semi-immune status was attained, it was observed that the two strains (Balb/c and CBA) exhibited different responses (Fig. 1). Prepatent period was longer for Balb/c than CBA, with Balb/c recovering fully from the parasitemia, and CBA did not. While mean %Hb reduction kinetics of Balb/c increased to a maximum on day 12 then dropped to a minimum on day 22, the mean %Hb reduction kinetics of CBA decreased continuously and in proportion to parasitemia level (Fig. 1). Interestingly, mean parasitemia was lower in Balb/c and higher in CBA (Table 1). The effect of parasitemia on Hb loss, calculated by the ratio mean Hb drop/mean parasitemia (Table 1) was more in CBA (lower ratio 15.9) than in Balb/c (higher ratio 28.7). The kinetic

Table 1 Magnitude of Hb reduction, peak reticulocyte count, and peak parasitemia in the semi-immune mice strain on the day minimum Hb was observed

Parameters	Balb/c	CBA	*p* value[a]
n	6	7	–
Mean Hb reduction,% (SD)	30.5 (29.7)	50.6 (10.5)	0.25
Mean Parasitemia,% (SD)	3.7 (3.2)	11.1 (9.7)	0.22
Mean Hb drop/mean parasitemia ratio (SD)	28.7 (25.6)	15.9 (12.9)	0.57
Mean peak reticulocyte count, % (SD)	8.5 (4.4)	7.3 (0.8)	0.76
Mean spleen size, cm (SD)	3.04 (0.11)	2.75 (0.13)	0.01

Hb reduction is the difference between maximum and minimum %Hb during the last cycle of infection without treatment as shown in Fig. 1. Each mouse showed minimum %Hb level (maximal reduction) on different days. The data presented here are values of one experiment

[a] Mann–Whitney test

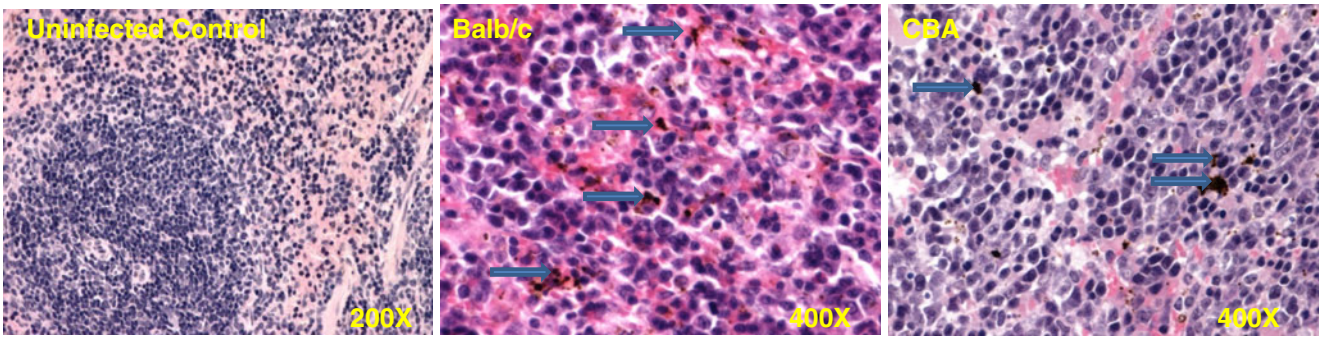

Fig. 2 Hematoxylin–eosin (HE) staining of spleen of uninfected control (n=3), semi-immune Balb/c (n=3), and CBA (n=7). The organs were removed when similar minimum %Hb reduction was observed as shown in Fig. 1 (Balb/c, days 18–22, and CBA, days 16–18). Sections of these organs were stained with HE and analyzed with light microscopy. The *blue arrows* indicate malaria pigment deposition in the spleen

profile of reticulocyte count (Fig. 1) revealed erythropoietic response to be slightly better in Balb/c with higher mean peak reticulocyte count of 8.5% and 7.3% in CBA, p=0.76 (Table 1). Splenomegaly was observed in both semi-immune mice, increasing in size between two and three times, than the uninfected mice. Increase in spleen size was significantly higher in Balb/c, p=0.01 (Table 1).

Histopathological study

Spleen Clear distinction between red and white pulp, resting follicles, and marginal zone was evident in the spleen of normal uninfected control mice (Fig. 2). The splenomegaly observed in all the infected semi-immune mice might be as a result of an overall enlargement of the red and white pulp (Martins et al. 2009) as shown in Fig. 2. In addition, the germinal centers in the spleen of semi-immune mice lost their typical structure. Malaria pigment deposition (indicated arrows) was observed in both strains. However, that of Balb/c was fine while that of CBA were aggregates as shown in Fig. 2. By means of the semi-qualitative approach, it was observed that between two and three thick/clumps of malaria pigments were seen at 400× magnification of the CBA spleen (Fig. 2), while smaller sizes of the malaria pigments were seen in that of Balb/c. Hypertrophy of the red pulp and intense proliferation of the cells in the resting follicles were also observed in the semi-immune mice.

Liver The liver sections of the normal uninfected control mice revealed normal, clear architecture, and color, such as sinusoids and Kupffer cells, as shown in Fig. 3. Hypertrophy of Kupffer cells are seen in all the semi-immune mice strains in addition to congested sinusoids (Fig. 3). Infected RBCs were also not observed, except for some few uRBCs. Meanwhile that of the infected immune mice showed some amount of malaria pigment deposition (indicated arrows) in the sinusoids. Semi-qualitative approach revealed five to eight malaria pigments thicker/clump in CBA. Those of Balb/c were few in number between 3 and 4, as shown in Fig. 3.

Brain It was also observed that the brain tissues of the normal uninfected control mice did not show any sign of RBC sequestration or leukocytes infiltration. Abnormal features, such as hemorrhages and edema, were not

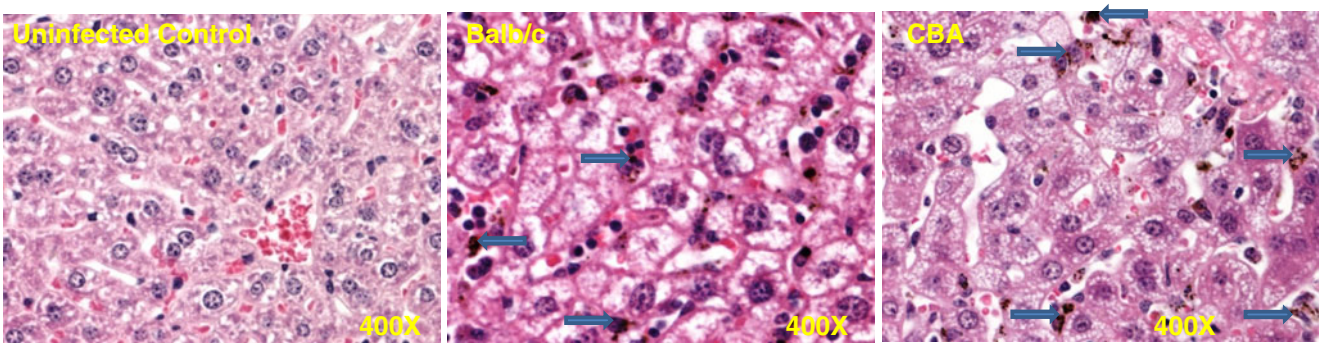

Fig. 3 Hematoxylin–eosin (HE) staining of liver of uninfected control (n=3), semi-immune Balb/c (n=3), and CBA (n=7). The organs were removed when similar minimum %Hb reduction was observed as shown in Fig. 1 (Balb/c, days 18–22, and CBA, days 16–18). Sections of these organs were stained with HE and analyzed with light microscopy. The *blue arrows* indicate malaria pigment deposition in the liver

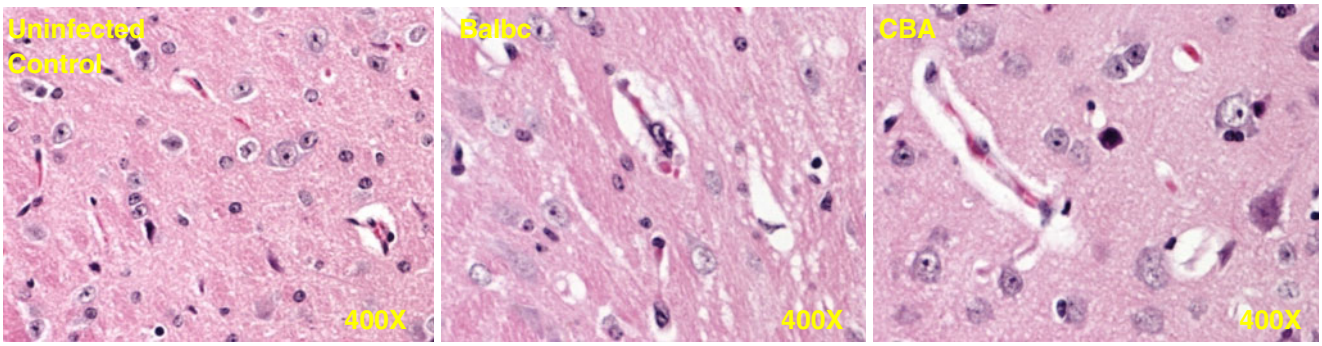

Fig. 4 Hematoxylin–eosin (HE) staining of brain of uninfected control (n=3), semi-immune Balb/c (n=3), and CBA (n=7). The organs were removed when similar minimum %Hb reduction was observed as shown in Fig. 1 (Balb/c, days 18–22, and CBA, days 16–18). Sections of these organs were stained with HE and analyzed with light microscopy

observed as shown in panels of the semi-immune mice strains (Fig. 4). Interestingly, the brain tissues of all the semi-immune mice strains did not show any endothelium-adherent hyperactivated macrophages.

Kidney The kidney tissues of the normal uninfected control mice showed normal kidney cells, with clear, clean glomeruli, and uRBC when compared with the semi-immune mice, as shown in Fig. 5. In addition, no malaria pigment deposition was observed.

Lung The lung tissues of normal uninfected mice show clear alveolar walls with some few uRBCs. The infected semi-immune mice on the other hand showed some alveolar wall thickening in both Balb/c and CBA, as shown in Fig. 6. Similar features such as normal RBCs (uRBCs) were also observed in the alveolar walls and blood vessels of all the mice strains.

Heart Uninfected control mice showed characteristic heart muscle tissues and with uRBC. And a group of uRBC can be seen in one of the chambers of the heart of Balb/c, as shown in Fig. 7. The histopathological finding of the heart muscles of CBA also shows similar features with no sequestration of iRBC (Fig. 7).

Muscle Similar characteristic features were observed in the normal uninfected control mice muscle tissues and the semi-immune mice strains, data not shown. No obvious sequestration of iRBC in the muscle fibers of both semi-immune mice strains was observed.

Discussion

This study describes the histopathological changes in some organs of semi-immune SMA model of mice. Variation in malaria pigment deposition has been observed in some of the organs of this semi-immune mice model. Even though *Pb*ANKA is regarded as a CM model, its use in the anemia studies of chronic situation (Evans et al. 2006; Helegbe et al. 2009) shows that this can be adapted for the study of SMA in the semi-immune.

Despite the fact that experimental animal models cannot reproduce all the features of human disease, it could be

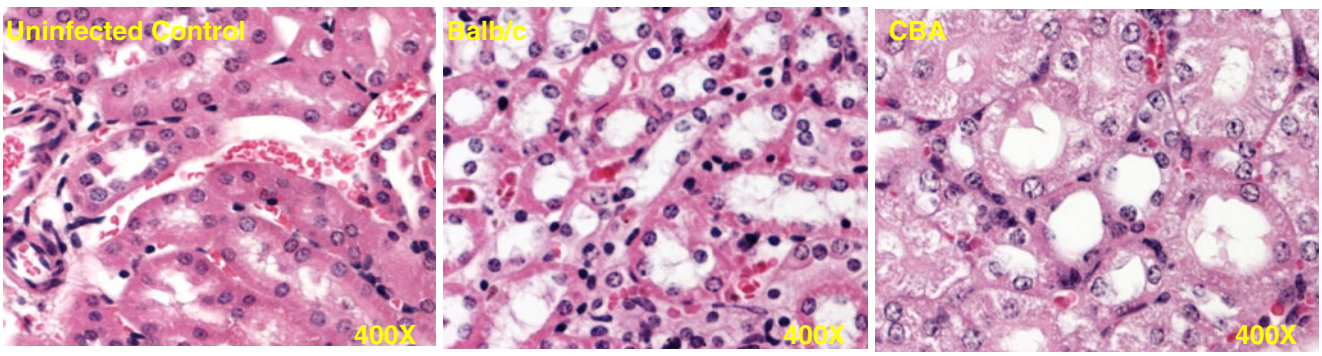

Fig. 5 Hematoxylin–eosin (HE) staining of kidney of uninfected control (n=3), semi-immune Balb/c (n=3), and CBA (n=7). The organs were removed when similar minimum %Hb reduction was observed as shown in Fig. 1 (Balb/c, days 18–22, and CBA, days 16–18). Sections of these organs were stained with HE and analyzed with light microscopy

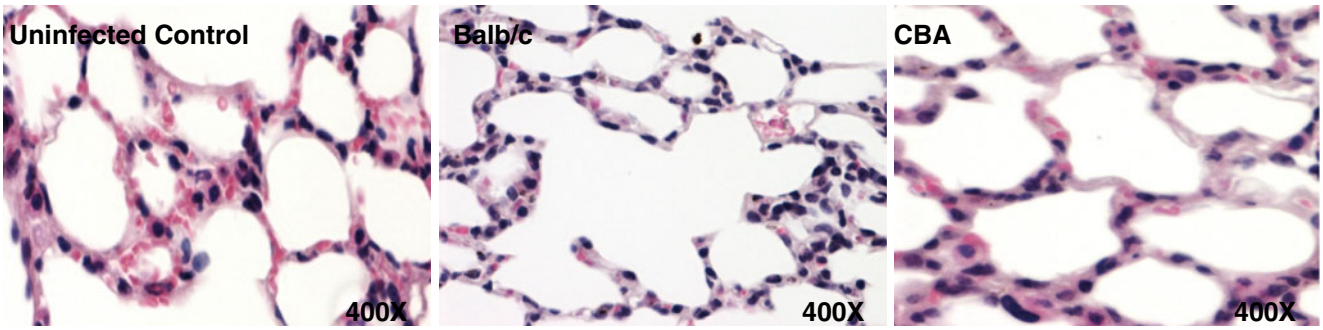

Fig. 6 Hematoxylin–eosin (HE) staining of lung of uninfected control (n=3), semi-immune Balb/c (n=3), and CBA (n=7). The organs were removed when similar minimum %Hb reduction was observed as shown in Fig. 1 (Balb/c, days 18–22, and CBA, days 16–18). Sections of these organs were stained with HE and analyzed with light microscopy

explored to explain some phenomena in human situations. Also animal studies have been useful in understanding the mechanisms of pathogenetic basis of various disease conditions to help in prophylactic or therapeutic interventions. Repeated infection plays a role in providing protection to the semi-immune mice strains where antibody titer was observed to be high (Helegbe et al. 2009). One of the roles of antibody during malaria infection is that it prevents the cytoadherence of iRBC onto the endothelial walls of the blood vessels (Druilhe and Perignon 1994). Since antibody titer was observed to increase in semi-immune individuals, it could be an explanation why sequestration was not observed in the brains of the semi-immune but occurred in the naïve. This might contribute to less CM incidence in adults (who have high antibody titer due to long exposure to plasmodium infections) than children in endemic areas. While sequestration in the adipose tissue, lung and spleen (Franke-Fayard et al. 2005), and brain hemorrhages (Amante et al. 2007) have been associated with severe malaria in naïve mice, none is reported in the histopathological sections of the organs of each semi-immune mice strain. Our study, however, did not evaluate any study on the naïve. In addition, the lack of endothelium-adherent hyperactivated macrophages in the brain, a hall mark of acute infection by *Pb*ANKA, could help explain the absence of systemic damage.

Splenomegaly, which was observed in all the infected semi-immune mice, might be a result of overall enlargement of the red and white pulp (Martins et al. 2009), suggesting hyperactivity of the macrophages. The significant difference in spleen size of the semi-immune mice strains might suggest differential immune response. Closely related to the high parasitemia in CBA is the high malaria pigmentation in the spleen and liver when compared with the other strain. This is consistent with other studies where a higher level of parasitemia results in higher amounts of free hemoglobin leading to free heme released (Gramaglia et al. 2006; Pamplona et al. 2007; Penet et al. 2006). Malaria pigment does not only stimulate TNF secretion, but also impair macrophage function (Turrini et al. 1993). Thus, this relatively higher malaria pigmentation in CBA could result in higher impairment of macrophage function than Balb/c. Hemozoin do persist in macrophages for some months. Thus, we do not exclude the fact that a considerable amount of the malaria pigment in the histological sections might have been released during

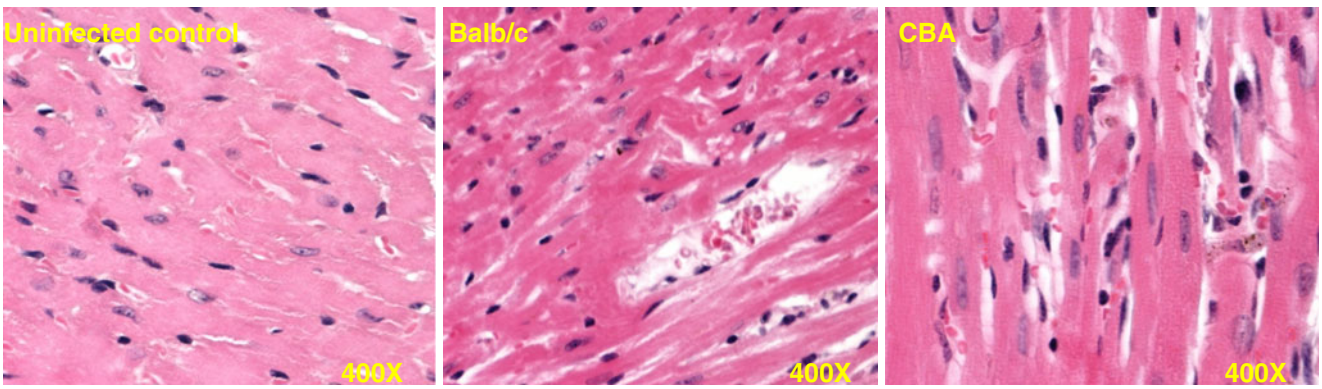

Fig. 7 Hematoxylin–eosin (HE) staining of heart of uninfected control (n=3), semi-immune Balb/c (n=3), and CBA (n=7). The organs were removed when similar minimum %Hb reduction was observed as shown in Fig. 1 (Balb/c, days 18–22, and CBA, days 16–18). Sections of these organs were stained with HE and analyzed with light microscopy

previous cycles. Our data presented here, the mean Hb loss/mean parasitemia ratio, being higher in Balb/c than CBA (Table 1), goes further to substantiate the assertion that more uRBC destruction may be involved in the Hb loss in Balb/c. While Hb loss in CBA may be due to direct lyses of iRBC due to its high parasitemia (Table 1), high antibody titer in the Balb/c as reported in an earlier study (Helegbe et al. 2009) enhances the sensitization of the RBC (both iRBC and uRBC) resulting in the significant Hb loss in the Balb/c (Evans et al. 2006; Helegbe et al. 2009). It is possible, polyclonal B cell activation and proliferation may be higher in Balb/c resulting in the high immunoglobulin production (Donati et al. 2004).

The inability to observe sequestration in the lungs might be due to the approach used in this study as compared to another (Franke-Fayard et al. 2005) where with the use of chemiluminescence sequestration was observed. Furthermore, the extent of malaria pigment deposition in the liver may help explain the hepatic dysfunction during *Pb*ANKA infection (De-Oliveira et al. 2006). However, we are not sure if malaria pigment deposition in the liver during *Plasmodium falciparum* infection (Guha et al. 2006; Kochar et al. 2003), can have any relation with oxidative stress induction as evidenced from reduced glutathione concentration, which correlated with degree of parasitemia (Guha et al. 2006). It is also not clear how malaria pigment influence hepatic dysfunction of these semi-immune individuals, but metabolic changes have been implicated in the late stage of naïve Balb/c (Penet et al. 2007). Overall, although these semi-immune mice strains seem highly vulnerable to anemia despite low parasitemias, they conversely appear to be quite protected from multi-organ pathology, which is a hallmark of infections in non-immune mice. Thus, further studies exploring the bone marrow activity in relation to these organs' histology need to be carried out to help explain better the anemia and RBC destruction at these low parasitemias of the semi-immune.

In conclusion, high Hb loss in the SMA model of the semi-immune in Balb/c at low parasitemia might be contributed mainly by uRBC, which was reflected by relatively low percent parasitemia and low malaria pigment deposition. This may therefore not be masked by sequestered iRBC in any of these organs as might be suspected.

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Competing interest There is no competing interest for any of the authors of the manuscript either due to commercial or other affiliations.

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