Acute and chronic histopathologic changes in wild type or TLR-2\textsuperscript{-/-}, TLR-4\textsuperscript{-/-}, TLR-6\textsuperscript{-/-}, TLR-9\textsuperscript{-/-}, CD14\textsuperscript{-/-}, and MyD-88\textsuperscript{-/-} mice experimentally infected with \textit{Plasmodium chabaudi}.

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Abstract

Malaria is caused by protozoan parasites of the genus \textit{Plasmodium}, which is transmitted by Anopheline mosquitoes. Experimental murine models using rodent malaria are useful for studying pathologic aspects of severe malaria. We evaluated histopathologic lesions of TLR-2\textsuperscript{-/-}, TLR-4\textsuperscript{-/-}, TLR-6\textsuperscript{-/-}, TLR-9\textsuperscript{-/-}, CD14\textsuperscript{-/-} and MyD88\textsuperscript{-/-} mice experimentally infected with \textit{Plasmodium chabaudi}. Frequencies and severity of microscopic lesions in the spleen and liver at one and four weeks post infection (wpi) were determined. At one wpi, adherence of macrophages to the endothelial surface was the most evident change, whereas at four wpi there was marked accumulation of cytoplasmic pigment in macrophages in the liver and spleen. Lesions were not markedly influenced by the absence of TLRs, MyD88 or CD14. Our findings suggest that acute and chronic phases of murine infection with \textit{P. chabaudi} are characterized by distinct lesions. In addition, TLRs and MyD88 are not essential to promote these lesions during \textit{P. chabaudi} infection.

Key Words: rodent malaria; \textit{Plasmodium chabaudi}, TLRs, MyD88, CD14; histopathology;

Introduction

Malaria is considered one of the most important infectious diseases worldwide. It is prevalent in tropical regions with approximately half of the world population at risk of infection, and more than one million annual deaths. Malaria is caused by protozoan parasites belonging to the genus \textit{Plasmodium}, which are transmitted by blood-feeding Anopheline mosquitoes (24). The disease is characterized by a range of clinical features from asymptomatic infection to a fatal disease (40).

Four rodent malaria parasite species, namely \textit{Plasmodium berghei}, \textit{P. vinckei}, \textit{P. yoelli}, and \textit{P. chabaudi}, have been used as powerful tools for elucidating some of the aspects of host-pathogen interactions in malaria as well as for \textit{in vivo} testing of newly developed therapeutic agents (3). The advantages of using laboratory mice as a model for malaria include a well studied immune system of the host, the opportunity to assess pathologic changes at all stages of the disease, and the availability of genetic variants (25). \textit{P. chabaudi} has been considered a good choice as an experimental model due to its life cycle, physiologic and biologic similarities to \textit{P. falciparum} (12), the most important causative agent of human malaria. These similarities include preferential invasion of mature erythrocytes, synchronicity of asexual blood forms, and a delayed formation of gametocytes until late in the infection cycle (10). In addition, drug resistance studies have demonstrated that \textit{P. chabaudi} and \textit{P. falciparum} have similar mechanism of resistance (11).
Histopathologic lesions often described in the liver and spleen of rodent with malaria include hypertrophy and hyperplasia of Kupffer cells containing malarial pigment and cytoplasmic cellular debris, portal mononuclear inflammatory infiltrates, and adhesion of macrophages to the endothelial surface of hepatic blood vessels. In the spleen, usually there are congestion and increased numbers of macrophages containing cellular debris and malaria pigment in the red pulp (3).

Hemozoin, a malarial pigment, is a polymer of heme produced by the parasite during hemoglobin breakdown inside the host red blood cells (RBC) (13). RCB lysis during infection results in release of merozoites with this pigment, which are phagocytized by circulating monocytes, neutrophils and resident macrophages (4). The amount of pigment in tissues increases throughout infection, so the greater amount of pigment, greater degree of chronicity of lesion (8). At first, hemozoin was considered only as a waste product of the parasite resulting from heme detoxification, however, several lines of evidence strongly suggest that it may play an important role in cytokine induction and parasite cytoadherence during malaria infection (20).

As chronic lesions in rodent malaria, cellular depletion either in B-dependent or T-dependent regions in the white pulp are observed. Also there is lymphoid hyperplasia with increased numbers of lymphocytes, both in the B and T zones of the spleen, and a decrease in reticular fibers in the liver (3).

There are some studies investigating the role of innate immune response components in rodent malaria (1, 14, 18, 22, 29, 31). Toll-like receptors (TLRs) are a family of pattern-recognition transmembrane receptors that play an important role in host defense, recognizing pathogen-associated molecular patterns during innate immune response (7). They act as host sensors of conserved structural components of viruses, bacteria, fungi, and protozoan (2, 6), signaling to adaptor molecules that triggers expression of proinflammatory cytokines and chemokines (39, 38). Plasmodium contains ligands that bind to at least three TLRs, including glycosylphosphatidylinositol anchors, which have been shown to bind to TLR-2 and lesser extent to TLR-4 (22), and DNA trapped within hemozoin, which can stimulate dendritic cells via TLR-9 (14, 31). CD14 is a glycosylphosphatidylinositol-anchored glycoprotein expressed on monocytes/macrophages, which binds LPS favoring its recognition by TLR-4. TLR-2 can heterodimerize with TLR-6, forming a type-1 transmembrane protein (36). MyD88, an intracellular adaptor protein for TLR-4 and other TLRs, is involved in the initial host response to erythrocytic stages of Plasmodium (1, 22, 31). CD14 has been described as a membrane receptor for several molecules, including peptidoglycan and lipoparabionmannan, which are ligands for TLR-2 (26).

Franklin et al. (2007) (18), investigating the role of different TLRs and MyD88 in host resistance to infection and rodent malaria pathogenesis, demonstrated that deficiency of CD14, TLR-2, TLR-6, TLR-4, or TLR-9 had no major effect on immunological control of parasite replication, cytokinemia and cytokine-mediated clinical signs associated with rodent malaria. However, MyD88-deficient mice showed TNF-α and INF-γ decreased production and attenuated clinical signs when compared to wild type. This finding was associated with blocking the activation of T cells during primary P. chabaudi infection by impaired production of these pro-inflammatory cytokines, thereby protecting the animals against malaria symptoms. In addition, Cadman et al. (2008) (9) suggest that changes in splenic microarchitecture during acute malaria is not dependent of TLRs and it is only mildly affected by MyD88 in mice. It is possible that stimulation of TLRs by Plasmodium may be required for full development of pathologic changes during the course of infection (9). In spite of previous studies addressing the role of TLRs in the innate immune response to rodent malaria, there have been no studies assessing pathologic changes in TLRs, MyD88 and CD14 knockout mice with experimental rodent malaria. Therefore, the aim of this study was to evaluate histopathologic changes in mice deficient in TLR-2, TLR-4, TLR-6, TLR-9, MyD88, and CD14, experimentally infected with P. chabaudi.

Material and Methods

A total of 135 mice, all backcrossed at least eight generations into the C57BL/6 background, were used in this study. Mice were inoculated intraperitoneally with 10^7 erythrocytes infected by P. chabaudi AS strain (5) and checked daily for survival, including wild type C57BL/6 (n=23 at 1 week post infection (1/πi), and n=23 at 4 weeks post infection (4/pi) respectively), MyD88^-/- (n=11 and n=6), TLR-2^-/- (n=9 and n=7), TLR-4^-/- (n=6 and n=4), TLR-6^-/- (n=8 and n=9), TLR-9^-/- (n=5 and n=7), and CD14^-/- (n=7 and n=10). Mice were subjected to euthanasia at one or four weeks after inoculation. Parasitemia was assessed by examining blood smears stained with Giemsa according to data published by Franklin et al. (2007), which demonstrated similar levels of parasitemia, when comparing MyD88^-/-, TLR2^-/-, TLR4^-/-, TLR6^-/-, TLR9^-/- and CD14^-/- to C57BL6 wild type mice. This study was approved by the Ethics Committee on Experimental Animal Use (CEUA/Fiocruz - program PO204-03).

Segments of the spleen, liver, lung, kidney, and heart were fixed by immersion in 10% buffered formalin during 24 hours. These samples were then dehydrated, and processed for paraffin embedding. Five µm sections were cut and stained with hematoxylin-eosin and Prussian blue.

A descriptive analysis of histopathologic changes of the spleen, liver, lung, kidney and heart was performed. Sections of the spleen and liver were subjected to blind microscopic evaluation to assess and score changes compatible with plasmodium infection such as macrophage adhesion to the endothelial surface of centrolobular veins in the liver, accumulation of pigments in the cytoplasm of Kupffer cells in the liver and macrophages in the red pulp of the spleen. To confirm that pigment inside cells was a result of hemoglobin breakdown (hemossiderin or hemozoin),
tissues were stained by Prussian blue, distinguishing them from others endogenous pigments. Intensity of pigment accumulation was assessed by measuring the area of pigment in digital micrographs of liver and spleen obtained from five randomly selected microscopic fields from each section, which were analyzed using the NIH Image 1.60 software.

Fisher’s exact test was used to compare frequency of lesions between different time points (1 and 4 weeks post-infection) or between parental wild type (C57BL6) and TLRs, MyD-88 or CD14 knockout mice. Student-Newman-Keuls’ (SNK) test was used to compare areas of pigment in histological sections. Differences were considered significant when p<0.05.

Results

Regardless of the genetic background of the mouse, the most significant lesions observed in the liver were accumulation of brown pigments, interpreted as hemozoin and/or hemosiderin in the cytoplasm of Kupffer cells located in the capillaries and sinusoids, adhesion of macrophages to the endothelial surface of centrolobular veins, and a predominantly periportal mild lympho-histiocytic infiltrate. In the spleen, there was an increased number of macrophages, including macrophages loaded with cytoplasmic brown pigment in red pulp, there was also mild to moderate lymphoid hyperplasia and/or focal or multifocal lympholysis, with morphological features of apoptosis.

Adhesion of macrophages to the surface of endothelial cells in centrolobular veins (Fig 1A), which was one of the most common lesions observed in liver, was more frequent at one than on four weeks post-infection in TLR-2\(^{-/-}\), TLR-6\(^{-/-}\), TLR-9\(^{-/-}\), and also C57BL6 mice (Fig.2). However, there were no statistical differences in frequencies of lesions comparing TLRs, MyD-88 and CD14 knockouts to parental wild type C57BL6 mice.

Figure 1. TLR2\(^{-/-}\) C57BL6 mice experimentally infected with Plasmodium chabaudi. (A) Liver with marked adhesion of macrophage to the endothelium (large arrow), and a small amount of brownish cytoplasmic pigment in Kupffer cells, at 1 week post infection. (B) Liver with a large amount of brownish cytoplasmic pigment in Kupffer cells (large arrows), and absence of macrophage adhesion to the endothelium, at 4 weeks post infection. (C) Spleen with a small amount of brownish cytoplasmic pigment in macrophages (large arrow), at 1 week post infection. (D) Spleen with a large amount of brownish cytoplasmic pigment in macrophages, at 4 week post infection. Hematoxylin and eosin.
Figure 2. Frequency (%) of macrophage adhesion in centrilobular veins of the liver from knockout mice at one week post-infection (1 wpi) and four weeks post-infection (4 wpi). Asterisk indicates that difference is statically significant (p<0.05), comparing frequency into two different times of infection. There was not statistical difference (p<0.05) comparing different TLRs, MyD-88 and CD14 knockout mice to C57BL6. Fisher’s Exact test. The letter n represents the total number of animals analyzed in each group.

Accumulation of brownish pigment in the cytoplasm of Kupffer cells (Fig. 1B) was observed in all groups of mice at 1 week post infection, as well as at 4 weeks post infection. This lesion tended to be more frequent at 4 weeks when compared to 1 week post infection, with statistically significant differences in TLR-2/- and CD14/- mice. There was not significant statistical difference in the frequency of hemoxzin and/or hemosiderin accumulation when wild type C57BL6 was compared to the knockout mice (Fig. 3A). Cytoplasmic pigment accumulation in macrophages was assessed by measuring the area of pigment in digital micrographs in liver and in spleen. Accumulation of pigment in the cytoplasm of Kupffer cells was more severe at 4 than at 1 week post infection in C57BL6, TLR2/-, TLR9/-, and CD14/- mice (Fig. 3B). However, there was no statistical difference in severity of pigment accumulation between wild type and knockout mice at same time point of infection.

In the spleen, there was mild accumulation of cytoplasmic pigment in macrophages in nearly all mice at one week post infection, and a more prominent accumulation at four weeks post-infection (Fig. 1C-D). Nevertheless, a lower frequency of this lesion in CD14/- mice comparing to C57BL6 mice at 1 week post infection was observed (Fig. 4A). In the spleen, we demonstrated that accumulation of brown pigment in macrophages of the red pulp was more severe at 4 than at 1 week post infection only in wild type C57BL6 and TLR2/- mice. We observed a lower intensity of this lesion in CD14/- mice at 1 week post infection when compared to wild type C57BL6 at the same time point (Fig 4B). Brown pigment in liver and spleen was stained with Prussian blue to identify hemosiderin pigment, then, we observed that its intensity on the evaluated tissues remained proportional to score data demonstrated in hematoxylin-eosin staining.

Figure 3. (A) Frequency (%) of brown pigment in the cytoplasm of Kupffer cells in liver of knockout mice at one week post-infection (1 wpi) and four weeks post-infection (4 wpi). Asterisk (*) indicates statistically significant differences (p<0.05), comparing frequencies from two different times of infection. There was no statistically significant differences (p>0.05) comparing different TLRs, MyD-88 and CD14 knockout mice to C57BL6. Fisher’s exact test. The letter n represents the total number of animals analyzed in each group. (B) Average of area occupied by brown pigments measured in pixels at one (1 wpi) or four weeks post-infection (4 wpi). Asterisk (*) indicates that difference is statistically significant (p<0.05), comparing frequency into two different times of infection. There was not statistical difference (p>0.05) comparing different TLRs, MyD-88 and CD14 knockout mice to C57BL6. SNK’s test. The letter n represents the total number of animals analyzed in each group.

Our data, demonstrates the kinetics of lesions in rodent malaria, which could be grouped into two phases: an acute phase when predominates adhesion of macrophages to endothelial cells (at 1 week post infection), and a chronic phase characterized by accumulation of cytoplasmic pigment in macrophages in the spleen and liver (4 weeks post infection), which is in good agreement with previous findings reported by Andrade Jr et al., 1991 (3).
Silva et al., Acute and chronic histopathologic changes in wild type or TLR-2\(^{-/-}\), TLR-4\(^{-/-}\), TLR-6\(^{-/-}\), TLR-9\(^{-/-}\), CD14\(^{-/-}\), and MyD-88\(^{-/-}\) mice experimentally infected with *Plasmodium chabaudi*. Braz J Vet Pathol, 2011, 4(1), 5-12.

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**Discussion**

Ours findings suggest that TLRs are not critical to promote histopathologic changes associated to *P. chabaudi* infection in mice, since we did not detect any significant differences in frequency or degree of severity when parental mice were compared to TLRs knockout. These findings are supported by previous reports that demonstrated that TLR-independent pathways also play a role inducing inflammatory responses in malaria (15).

The lack of CD14, a co-receptor that enhances activation of TLR-2 and TLR-4 (26), was associated with a decreased intensity of hemosiderosis in the spleen at one week post infection. However, this finding was not observed in the liver. It could be explained because *Plasmodium* blood stage infection suppresses CD8 T cell responses against the liver stage of the parasite. Therefore, the blood stage of the parasite inhibits the establishment of a protective response against the initial liver stage (28).

Adhesion of macrophages to the endothelium was observed in both wild type and knockout mice, mostly at 1 week post infection, indicating that the establishment of this lesion does not require TLRs 2, 4, 6 and 9, MyD-88, or CD14. Several endothelial cell proteins have been identified as potential receptors for vascular adhesion molecule-1 (VCAM-1), and endothelial leukocyte adhesion molecule-1 (ELAM-1) in humans infected with *P. falciparum* (30). These proteins may be responsible for adherence of macrophage in blood vessels in a *P. chabaudi* infection. Moreover, this lesion was more frequent in the acute phase of infection and in the endothelium of hepatic centrallobular veins. These results are in good agreement with the notion that in initial stages of malaria, infected red blood cells (RBCs) activate endothelial cells, thus triggering a localized inflammatory response (19, 37). Similar lesions were observed in experimental infection of *P. yoelii nigeriensis* in CBA/J mice, including macrophage adhesion to the endothelium in the liver, mononuclear infiltrate in the sinuses, and macrophages with hemosidn in liver and spleen (34).

*Plasmodium* infects and causes lysis of RBCs and degradation of hemoglobin (16). During the acute phase of malaria infection the parasite grows exponentially to a high density through replication in RBCs, and subsequently there is a decline in the growth rate. Different malaria strains reach different peak densities in this phase. The dynamics then become much more complex, and are strongly influenced by the interplay between specific immune responses and antigenic variation which allows the parasite to evade these specific responses (33, 32). During the chronic phase of infection, there is a marked destruction of infected RBCs, which explains the increase of cytoplasmic pigments in macrophages in the liver and spleen at 4 weeks post infection. Prussian blue staining, confirmed that the pigment observed contained iron.

Our findings that histopathological changes are not markedly influenced by the absence of TLRs, MyD88 or CD14 are somewhat unexpected considering that two putative activators of cytokine production during *Plasmodium* infection includes the malarial glycosylphosphatidylinositol (GPI) anchors and parasite-derived DNA bound to hemozoin that activate TLR-2 or TLR-9, respectively (22, 29). Apparently in humans TLR-2, TLR-9, and MAL/TIRAP are involved in innate immune response to malaria (24, 21). Importantly, Franklin et al. (2007) studying the same experimental mice used in this study observed that MyD88 in coordination with these TLRs and coreceptors (CD14) plays an important role in systemic production of pro-inflammatory cytokines like TNF-\(\alpha\) and INF-\(\gamma\), during acute phase of malaria, although they
are not critical for the immunological control of \textit{P. chabaudi} infection, which is compatible with ours results indicating that these genes do not significantly interfere with adhesion of macrophages to endothelial cells. Conversely, absence of CD14 resulted in a decrease frequency of accumulation of cytoplasmic pigment in splenic macrophages at 1 week post infection, which may be associated with the role of CD14 as co-receptor (26) that recognizes microbial ligands, resulting in a potent inflammatory cascade, which induces release of TNF-α and IL-1β (27).

In conclusion, our findings demonstrated that acute and chronic phases of murine infection with \textit{P. chabaudi} are characterized by lesions in the spleen and liver, whereas TLRs 2, 4, 6 and 9, and MyD-88 are not essential for the development of these lesions. In addition, the present data demonstrate a possible role of CD14 in host defense against \textit{Plasmodium chabaudi} since lack of CD14 resulted in a decreased intensity of accumulation of cytoplasmic pigments in splenic macrophages during the acute phase of infection.

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