International Scholars Journals

Advanced Journal of Microbiology Research ISSN 2241-9837 Vol. 13 (3), pp. 001-005, March, 2019. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Cytokine genes expression in mice hepatocytes during malaria infection

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Accepted 11 February, 2019

The aim of this study was to investigate the inflammation genes (IL-1 , IL-6, TNF - and iNos) responses to *Plasmodium chabaudi* malaria in the liver of female C57BL/6 hepatocytes via mRNA expression due to infection with *P. chabaudi* at different time points. Mice were injected intraperitoneally (ip) with 10^6 *P. chabaudi*-infected erythrocytes and then scarified at days (0, 1, 4 and 8 respectively). RT-PCR was used to quantify liver inflammation genes. The levels of IL-1 , IL- 6 and TNF were significantly increased at days 1 and 8. The total iNos were significantly increased at all days after infection. In conclusion, present data has shown that infection with *P. chabaudi* stimulated infalammation genes in the liver. Thus, we suggest the implication of oxidative stress due to outcome of malaria in mice hepatocytes according to its natural function need to be confirmed with a larger number of samples to be used as a reliable inflammation detection method.

Key words: Inflammation, malaria, mice, liver.

INTRODUCTION

Despite decades of intense research, malaria remains a major global health problem with an estimated mortality of 1-2 million (Alkahtani, 2011). Due to increasing the resistance of parasites no effective vaccine is available and all efforts to develop an effective anti-malaria vaccine have failed to date (Bergmann-Leitner et al., 2009; Alkahtani, 2010). However, several vaccine candidates and vaccine platforms have yielded encouraging results in animal models of malaria, but have not been sufficiently evaluated in clinical trials. A rather convenient malaria model system is the *Plasmodium chabaudi* blood stage infection in mice, since *P. chabaudi* shares several characteristics with *P. falciparum*, the most dangerous human malaria species (Hernandez-Valladares et al., 2005).

Liver plays a very important role in malaria: It is the site of pre-erythrocytic development of *Plasmodium* parasites, and also acts as an effector against malarial blood stages as recently shown (Krücken et al., 2005b; Wunderlich et al., 2005). Kupffer cells are able to eliminate parasite-derived hemozoin and even *Plasmodium*-infected erythrocytes. As a result of this process, Kupffer cells become increasingly activated, which manifests itself as a production of NO (nitrogen oxide), diverse ROS (radical oxygen species), and the cytokines IL-1 and TNF . In addition, cytokines have primary roles in physiology, including neurophysiology, innate and acquired immune responses and wound healing, as well as pathogenesis. Some cytokines, such as TNF, LTs and IL-1 are pro-inflammatory, whereas others such as IL-4, IL-10 and the TGF family, are anti-inflammatory, being subsequently induced to inhibit these changes (Clark et al., 2010). NO, ROS and the cytokines result in enormous pathological complications in the liver. Indeed, IL- 1 and TNF are thought to induce the acute phase response and a dramatic decrease in liver metabolism, both majorly

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based on liver cells (Krücken et al., 2005a; Guha et al., 2006). Recent results with deficient mice in the expression of those cytokines indicate that they are in general more sensible to insults resulting in neural damage. Some of the actions induced by TNF- and IFN-

including both beneficial and detrimental, are mediated by inducible nitric oxide synthase (iNOS) -derived nitric oxide (NO) production (Munoz-Fernandez and Fresno., 1998).

The present study was undertaken to investigate if the inflammation genes responses to *Plasmodium chabaudi* malaria in the liver of mice and to detect if these genes could be a good marker for inflammation and metabolism instability due to infection with *Plasmodium chabaudi* in mice hepatocytes.

MATERIALS AND METHODS

Animals and infection

Normal female C57BL/6 mice 10-14 weeks old were obtained from the central animal facilities of Heinrich Heine University, Düsseldorf, Germany, and housed in plastic cages. Mice were bred and maintained under specified pathogen free conditions. Mice were fed with standard diet (Wohrlin, Bad Salzuflen, Germany) and water *ad libitum*. The experiments were approved by the State authorities and followed German law on animal protection.

Blood stage malaria

A non-clonal line of *P. chabaudi* was used (Wunderlich et al., 1988) exhibiting a very similar, but not identical restriction length polymorphism pattern to *P. chabaudi* (Kruecken et al., 2005b). Erythrocytic stages of *P. chabaudi* were passaged weekly in NMRI mice. From these mice, blood was taken and 10^6 *P. chabaudi*-infected erythrocytes were injected i.p. in the mice. Parasitemia was evaluated in Giemsa-stained blood smears. The total number of erythrocytes was determined in a Neubauer chamber. A total of five mice were scarified at each day (0, 1, 4 and 8 respectively) by cervical dislocation. Livers were removed and cut into smaller pieces and kept at -80°C.

RNA-Isolation

Approximately 250 mg frozen liver was homogenized with an ultraturrax in 5 ml Trizol (Peqlab Biotechnology, Erlangen, Germany) for one minute. After mixing with 1 ml chloroform for 15 s, the suspension was incubated for 15 min at room temperature and centrifugated at 3.000 x g for 45 min. After isopropanol precipitation of the supernatant, the pellet was washed twice with 80% ethanol and air-dried and dissolved in 200 µl RNase-free water. RNA concentrations were determined at 260 nm, and the purity of RNA was checked in 1% agarose gel.

Quantitative real-time PCR

All RNA samples were treated with DNase (Applied Biosystems, Darmstadt, Germany) for at least 1 h and then converted into cDNA following the manufacturer's protocol using the Reverse Transcription Kit (Qiagen, Hilden, Germany). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the ABI Prism® 7500HT Sequence Detection System (Applied Biosystems, Darmstadt, Germany) with SYBR Green PCR Mastermix from Qiagen (Hilden, Germany). We investigated the genes encoding the mRNA for following proteins: 18S, Interleukin-1beta (IL-1), Tumor necrosis factor alpha (TNF), Interleukin-6 (IL-6), Inducible nitric oxide synthase iNOS. All primers used for qRT-PCR were commercially obtained from Qiagen. PCR reactions were conducted as follows: 2 min at 50°C to activate uracil-N-gylcosylase, 95°C for 10 min to deactivate UNG, 40 cycles at 94°C for 15 s, at 60°C for 35 s and at 72°C for 30 s. Reaction specificity was checked by performing dissociation curves after PCR. For quantification, mRNA was normalized to 18S rRNA. The threshold Ct value is the cycle number selected from the logarithmic phase of the PCR curve in which an increase in fluorescence can be detected above background. The Ct is determined by subtracting the Ct of 18S rRNA from the Ct of the target (Ct = Ct-target – Ct-18S rRNA). The relative mRNA levels of non-infected mice are described as a ratio of target mRNA copy to 18S rRNA copy = 2^{-Ct}. The fold induction of mRNA expression on days (0, 1, 4 and 8 *p.i.* was determined using the 2^{-ct}-method (- ct = ct day 0 *p.i.* - ct day 8 *p.i.*).

Blood analysis

In sera, IL-1 , IL-6, TNF were measured using ELISA kits (R&D Systems, Minneapolis, USA) according to the manufacturer's protocols. Total iNOS was analyzed using a commercially available kit (R&D Systems).

Statistical analysis

Two-tailed Student's t-test and Fisher's exact test were used for statistical analysis.

RESULTS

Characteristics of *P. chabaudi* infection

The parasitemia of mice with 10^6 *P. chabaudi*-infected erythrocytes became evident on day 4 and rocketed to reach its peak (48%) on day 8 and then reduced rapidly to about 0.4% on day 12 (Figure 1).

Inflammation genes expression

Quantitative real- time PCR was used to detect changes in mRNA levels of different inflammation genes. The levels of IL-1 , IL-6 and TNF were significantly ($P<0.01$) increased at day 1 and 8 compared with day 0 (Figure 2). The total of iNOS as a marker of oxidative stress was significantly ($P<0.01$) increased at all days after infection compared with day 0 and more expressed compared with the other genes (Figure 2).

Blood parameters

The data showed that infection caused strong significant ($P<0.01$) increases in the levels of cytokines IL-1 , IL-6

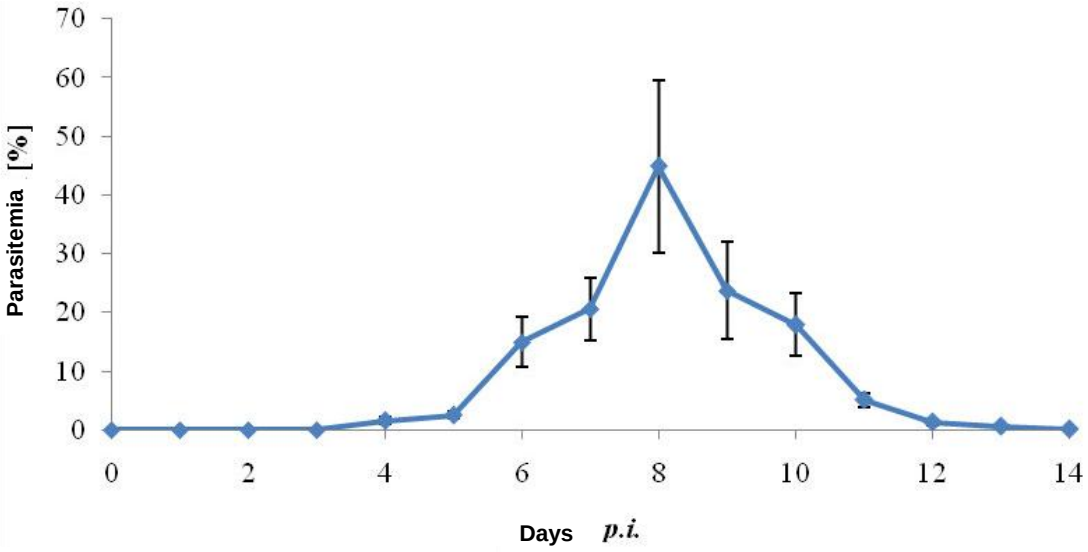

Figure 1. Parasitemia (48%) of female C57BL/6 mice (n=20) infected with 10^6 *P. chabaudi*-infected erythrocytes.

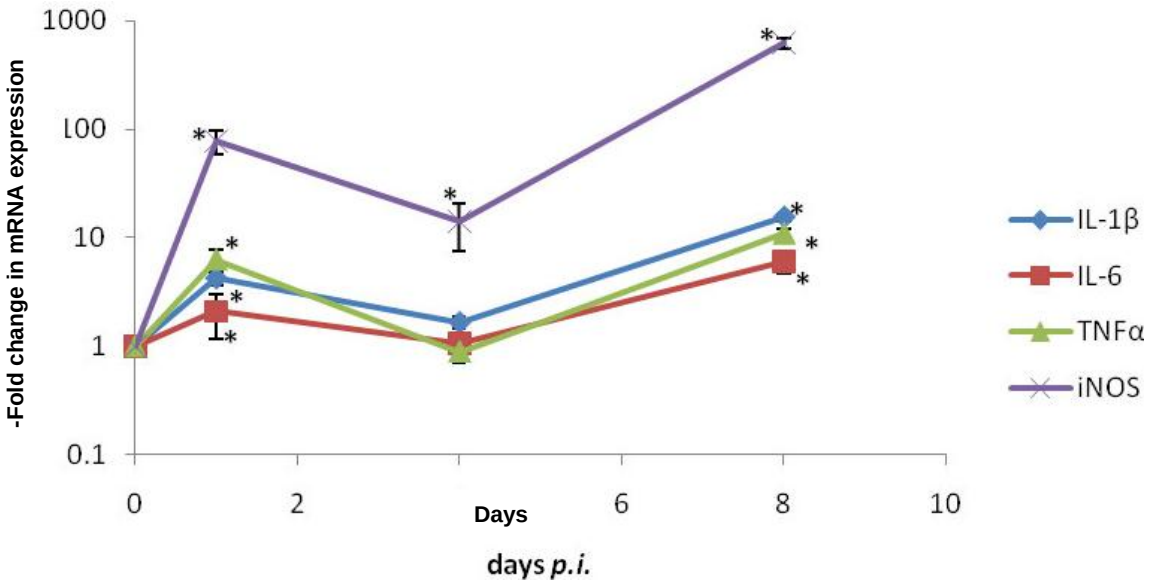

Figure 2. RT-PCR of IL-1 , IL-6, TNF and iNOS genes expression in the liver of *Plasmodium chabaudi* infected mice. The expression of these genes was measured at different times. The data present are the mean ±SE (n=5). *: significant value at (P<0.01).

(ng/l) and total iNOS (µmol/l) at peak parasitemia on day 8 *p.i.,* (Table 1).

DISCUSSION

Cytokines have essential roles in pathogenesis and they operate in networks and cascades to regulate cellular activities (Clarka et al., 2008; Clark et al., 2010). The obtained data showed that the infection with *Plasmodium chabaudi* caused liver dysfunction as shown by measuring the inflammation genes expression at different time points. As expected, infections caused increases the levels of these cytokines in liver especially at the peak of parasitemia but our result showed two peaks of gene expression on day 1 because of the acute phase response and another strong one on day 8 because of the peak parasitemia. About 80-90% of total macrophages reside as Kupffer cells in the liver, in particular in the periportal area supervising invasion of pathogens (Häussinger and Schliess, 2008). The increased release of TNF and IL-1 may induce local inflammatory

Table 1. Serum parameters of control (day 0) and infected (day 8) mice with 10^6 *P. chabaudi.*

Parameters in blood sera	0 day	8 days *p.i.*	*p*-value 8d *p. i.* vs. 0d
Parasitemia	0.00 %	48 %	< 0.01
IL-1 [ng/l]	1.17 ± 0.2	4.5± 1.3	<0.01
IL6 [ng/l]	1.3 ± 0.1	35.5± 10.2	< 0.01
TNF- [ng/l]	19.5 ± 0.6	26.5 ± 5.2	0.46
Total NOx [µmol/l]	28 ± 2	111 ± 7	< 0.01

responses, which may be associated with more pronounced liver injuries in infected mice (Barua et al., 2001).

On the other hand, TNF have many homeostatic physiological roles, which are involved in innate immunity, and cause inflammation when in excess (Clark et al., 2010). In addition, it has been published that proinflammatory cytokines, tumour necrosis factor (TNF)-, interferon (IFN)- and interleukin (IL)-6, have multiple effects in the central nervous system (CNS) not strictly cytotoxic being involved in (CNS) disorders, proliferation, differentiation, survival (Munoz-Fernandez et al., 1998). Recent results with deficient mice in the expression of those cytokines indicate that they are in general more sensible to insults resulting in neural damage. Some of the actions induced by TNF - , and IFN- , including both beneficial and detrimental, are mediated by inducible nitric oxide synthase (iNOS)-derived nitric oxide (NO) production. NO produced by iNOS may be beneficial by promoting the differentiation and survival of neurons. IL-6 does not induce iNOS, explaining why this cytokine is less often involved in this dual role protection and pathology (Nussler et al., 1995; Clarka et al., 2008).

However, TNF is highly pleiotropic. It is, for example, an *in vitro* tumor killer, a common regulator of normal physiology, including of synapses, a homeostatic agent in cell proliferation a key mediator of innate immunity and the master cytokine that precipitates the inflammatory response (Clark et al., 2010). Moreover, IL-1 is induced by TNF, and shares many of its activities, including the capacity to induce IL-6. This cytokine is often used as a marker for systemic inflammatory reactions because it appears in the circulation conveniently later, when illness is undeniable, and stays high for longer. However, both TNF and IL-1 induce IL-8, the prototype chemotactic cytokine, or chemokine, terms given to cytokines that attract cells along their concentration gradients. Here the pathology of malaria infections is caused by over-exuberant production of the mediators of innate immunity (Clark et al., 2010).

The increased of inflammation genes expression could be stimulate apoptosis process as a result of a wide variety of stimuli (Bergmann-Leitner et al., 2009; Alkahtani, 2011). The data presented here connect with previous results that malaria infection significantly induces liver apoptosis mediated by oxidative stress mechanisms (Guha et al., 2007). Apoptosis of host cells not only governs the further development of intracellular parasites but also is critical for the occurrence of pathology. Infection with mouse- virulent strains of *Toxoplasma* (i.e. those which display LD_{50} of less than 10 parasites, e.g. RH) leads to extremely elevated serum levels of the pro- inflammatory cytokines IFN- , IL-12, TNF- and IL-18 which coincides with exaggerated apoptosis of host cells. In addition other forms of leukocyte apoptosis including cell death induced by TNF- and IFN- certainly also contribute to the depletion of immune cells following infections with various protozoa including *Plasmodium* spp., *Leishmania* spp., *T. cruzi*, *T. gondii* and *C. parvum*. Importantly, such leukocyte death during parasitic infection is considered a critical parameter for the course of infection (Clarka et al., 2008; Alkahtani, 2011).

Conclusion

Under our experimental conditions, *Plasmodium chabaudi* induces up regulation of inflammation genes in the liver via IL-6, IL-1 , TNF and iNOS tests. Thus, we highly recommend confirming this result by measuring protein levels to be used as a reliable inflammation detection method.

ACKNOWLEDGMENT

The authors extend their appreciation to the Deanship of Scientific Research at King Saud University for funding the work through the research group project No. RGP-VPP-018.

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