

Original Research Article

**Variation of Mice Immune Responses After Infection with Lethal Strain *Plasmodium yoelii*:
Role of Inflammatory and Anti-inflammatory Cytokines**

ABSTRACT

Objective: Variability in resistance to infections is a well-known phenomenon that has been ascribed to genetic and immunologic factors. The present study was conducted to identify the mRNA expression pattern for some cytokines in two groups of mice from different genotypes.

Methods: Two groups of mice were used (C57BL/6 (B6) and DBA/2 mice). The mice were infected with lethal strain of *Plasmodium yoelii* 17XL (*P. yoelii* 17XL). Analysis of mRNA for Th1-type cytokines [Interferon- γ (IFN γ), Interleukins (IL)-12, IL-6, and tumor necrosis factor- α (TNF α)] and Th2-type cytokines [IL-4 and IL-10], was conducted using Reverse Transcription (RT)-PCR.

Results: The main difference was the significant rise in IL-12 and IL-6 in DBA/2 mice compared to their baseline value in B6 mice. The production of TNF α increased in the severe phase in B6 mice, in sharp contrast to its dominant production in the recovery phase of DBA/2 mice. The expression of anti-inflammatory cytokines, particularly IL-10, increased in DBA/2 mice. The production of immunoglobulin M (IgM) and IgG-type anti-DNA autoantibody was evaluated.

Conclusion: This study aimed to clarify the complexities of cytokines responses and their role in controlling malaria, and to present necessary data for the development of effective malaria vaccine and new anti-malaria drugs.

Keywords: Malaria, Cytokines, Mice

1. INTRODUCTION

Malaria, caused by parasites of the genus *Plasmodium* remains one of the leading causes of morbidity and mortality in the world. WHO estimated over 200 million cases and approximately 600 thousand deaths of malaria every year [1]. Efforts to control malaria have met a lot of difficulty; mosquito vectors have become increasingly resistant to insecticides, and malaria parasites are developing resistance to drugs due to their complex multi-stage life cycle. The parasite not only expresses a great variety of proteins, but these proteins also keep changing [2, 3]. Thus, new measures for malaria control are strongly needed to develop malaria vaccines. However, the complex interaction of parasite proteins with the immune system of the host has made it difficult to improve an efficient vaccine against the disease. Up till now, no vaccine formulation with sufficient efficacy against malaria parasite has been developed [4].

Extensive research of the immune response against malaria parasites has provided a wealth of information about the cells and cytokines involved in the pathophysiology of either the survival or fatal outcome of this infection [5, 6]. Malaria, like other human diseases, is driven by the production of cytokines, the inflammatory cytokines such as IFN γ , IL-12, IL-6, TNF α and anti-inflammatory cytokines, including IL-4 and IL-10. The early and intense secretion of pro-inflammatory T-helper 1 (Th1) cytokines mediated the successful resolution of infection that promotes parasite killing by macrophages and prevents immune-mediated damage [7]. This is subsequently suppressed by anti-inflammatory cytokines that mainly act by inhibition of the production of pro-inflammatory cytokines [8]. A proper balance between pro-inflammatory and anti-inflammatory immune response is necessary to control the pathogenesis of severe malaria [9, 10].

Studies on murine malaria suggested that resistant and susceptible mice infected with

Plasmodium are associated with different outcomes. Different strains of inbred mice infected with diverse species of plasmodia provoked distinctive immune responses [11, 12]. The immunologic responses that contribute to resistance vs susceptibility to parasitic infection seem to depend upon the presence of functionally distinct CD4⁺T cells: Th1 and Th2 cells [13]. Differential activation of CD4⁺ T-cells and resultant cytokine activity in malaria have been well defined in murine models. Cytokine production (TNF- α and IFN γ) appears necessary for the inhibition of parasitemia and stimulation of phagocytosis to enhance the clearance of parasitized erythrocytes [14, 15]. Intra-peritoneal injection of mice with recombinant IL-12 appears to induce IFN- α -mediated protection against *Plasmodium yoelii* sporozoite challenge, and IL-12 injection before sporozoite challenge was found to protect monkeys against malaria [16, 17]. Our previous results indicated that both Th1 and Th2 subsets of T helper lymphocytes are activated during *Plasmodium yoelii* non-lethal infection using different strains of mice with different genetic backgrounds [11, 18]. Other studies mentioned that regulatory T cells (tregs) might serve as an important regulator in blood-stage malaria infection [19- 21].

Along with T cells, cytokines produced by natural killer (NK) cells and macrophages contribute either to the resolution of malaria infection or to a persistent increase in concentrations of malaria parasitemia which leads to complicated malaria [22, 23]. However, we, as well as other investigators, have reported that protection against malaria is an event of innate immunity mediated by intermediate T (TCR^{int}) cells due to its feature as an intracellular pathogen [11, 24, 25]. Thus, the cytokine profile indicated a Th0 type in the early phase of malaria infection and a Th2 type in the late phase [18]. In the present study we sought to further understanding of the modulation of cytokine responses in resistant DBA/2 mice and to explore the characteristics of specific immune responses against blood stage *Plasmodium*.

2. METHODOLOGY

2.1 Mice and malaria infection

Two groups of B6 and DBA/2 mice at the age of 8-10 weeks were used. Each group involved 16 mice; the mice were maintained at the animal facility of Niigata University (Niigata, Japan) under specific pathogen-free conditions. Ethical guidelines on the dealing with the experimental animals were respected during the study. *P. yoelii* 17XL (lethal strain) was used [26]. Parasites were maintained by regular in vivo passages in mice. The mice were infected by intra-peritoneal (ip) injection with 10^4 parasitized erythrocytes per mouse. Mice were sacrificed at the indicated days (days 0, 3 and 7 for B6 mice and days 0, 3, 7 and 20 for DBA/2 mice), and blood was obtained by cardiac puncture. Sera were collected and stored at -80°C for evaluation of antibodies and cytokines levels.

2.2 Determination of parasitemia and hematocrit value

Parasitemia was observed by Giemsa-stained thin smears of tail blood every 2 to 3 days throughout the experimental period. Packed cell volume (PCV) was determined by centrifuging heparinized blood obtained from mice tail in a capillary tube at 10,000 RPM for five minutes. This separates blood into two layers. Packed red blood cells Volume divided by the total volume of blood sample gives PCV. PCV was calculated by measuring the lengths of the layers as a tube was used.

2.3 Cell preparation

Hepatic mononuclear cells were isolated using a previously described method [27]. Briefly, the liver was removed, pressed through a 200-gauge stainless steel mesh, and suspended in Eagle's minimal essential medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 5 mM HEPES and 2% heat-inactivated newborn calf serum. After washing with the medium, the cells

were fractionated by centrifugation in 15 ml of 35% Percoll solution (Pharmacia Fine Chemicals, Piscataway, NJ) for 15 min at 424.8 g. The pellet was resuspended in erythrocyte-lysing solution (155 mM NH₄Cl, 10 mM KHCO₃, 1 mM EDTA-Na and 170 mM Tris, pH 7.3). The splenocytes were gained by pressing the spleen through a 200-gauge stainless steel mesh. Splenocytes were used after erythrocyte lysing.

2.4 RT-PCR for cytokines mRNA

Mice were sacrificed on the indicated days after infection. Total RNA was extracted from the liver tissues of B6 and DBA/2 mice [26]. To detect mRNAs of IFN γ , IL-12P40, IL-6, TNF α , IL-4 and IL-10, RNA was subjected to reverse transcription-using primers of these genes and such cDNA was further amplified by the PCR method [26]. Briefly, total RNA was prepared from (30 mg) samples from the liver tissues of B6 and DBA/2 mice by using Isogen (Nippon Gene, Tokyo, Japan). cDNA was synthesized using 5 μ g RNA with Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences, Piscataway, NJ) and Oligo (dT) 15 Primer (Promega, Madison, WI). PCR amplification of synthesized cDNA was conducted as previously described [28]. Briefly, PCR was performed in a mixture containing a 1X PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl) (BRL), 1.0 mM MgCl₂, 0.2 mM dNTP, 0.2 mM sense and antisense primers, 2.5 U Taq polymerase (BRL), and 1 mL cDNA template (80 mM). PCR amplification was performed by Gene Amp PCR System 9700 (Perkin Elmer, Norwalk, CA). Amplifications were done by running 30 cycles under conditions of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and elongation at 72°C for 2 min. PCR products together with markers were electrophoresed on 2% agarose gel and stained by ethidium bromide to check single-band products. Primers of IFN- γ , IL-12P40, IL-6, TNF- α , IL-4, IL-10 and G3PDH were

as follows: IFN- γ (sense 5'-AAT GAA CGC-TAC ACA CTG CA-3' and antisense 5'-TGA AGA AGG TAG TAA TCA GG-3'); IL-12P40 (sense 5'-CGT GCT CAT GGC TGG TGC AAA G-3' and antisense 5'-CTT GAT CTG CAA GTT CTT GGG C-3'); IL-6 (sense 5'-GAC TTC CAT CCA GTT GCC TTC TTG GGA-3' and antisense 5'-AGG AGA GCA TTG GAA ATT GGG GTA G-3'); TNF- α (sense 5'-ATG AGC ACA GAA AGC ATG ATC CGC-3' and antisense 5'-AAA GTA GAC CTG CCC GGA CTC-3'); IL-4 (sense 5'-CCA GCT AGT TGT CAT CCT GC-3' and antisense 5'-GTG ATG TGG ACT TGG ACT CA-3'); IL-10 (sense 5'-GGA CAA CAT ACT GCT AAC CGG-3' and antisense 5'-ATA TTT CGG AGA GAG GTA CA-3'); G3PDH (sense 5'-ACC ACA GTC CAT GAA ATC AC-3' and antisense 5'-TCC ACC ACC CTG TTG CTG TA-3'). G3PDH Primers were used to determine the integrity of the RNA preparation.

2.5 Serum levels of anti-DNA antibody

Blood samples were taken on the indicated days after infection. After centrifugation, sera were immediately aliquoted and stored at -80°C until tested. Measurements of IgM and IgG antibodies reacting with single-stranded (ss) DNA obtained with the enzyme-linked immunosorbent assay (ELISA) method were modified as previously described [29]. Standard sera were obtained from MRL-*lpr/lpr* mice (after the onset of disease) and arbitrarily determined to contain 100 units of anti-DNA antibody. The titer was measured as a percentage in comparison with standard sera.

2.6 Transaminase measurement

Serum glutamic pyruvic transaminase (GPT) activity was measured with a Transaminase CII-test Wako kit (Wako Pure Chemical, Osaka, Japan).

138 **2.7 Histology**

139 Liver tissues were fixed in 10% phosphate-buffered formalin and embedded in paraffin. Sections
140 4 mm in thickness were stained with hematoxylin and eosin.

141 **Ethical consideration**

142 The experiments were done in the animal house, Department of Immunology, School of
143 Medicine, Niigata University, Niigata, Japan. Animal house ethical committee approved them.
144 Animal handling protocols meet the standard international guidelines by the National Institutes
145 of Health guide for the care and use of Laboratory animals. All mice were maintained in
146 pathogen-free insulators. Human endpoints to reduce pain or distress in mice were used via
147 euthanasia. Mice were sacrificed using CO₂ after which the cervical dislocation method was
148 used.

149 **Statistical analysis**

150 Data were analyzed by One-Factor Analysis of Variance (ANOVA) or student t- test.

151 **3. RESULTS**

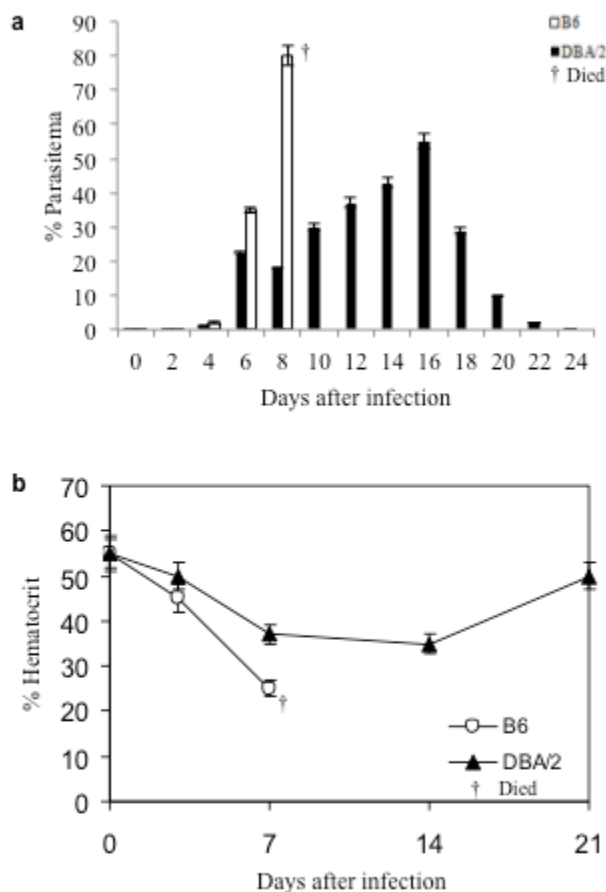
152 **3.1 Survival conditions of DBA/2 mice infected with malaria**

153 In both strains, the parasite appeared in the blood on day 3 after infection, parasitemia was very
154 high and the mice succumbed to death between day 7 and day 10 in B6 mice. In DBA/2
155 parasitemia extended to a peak of 60% on day 15 followed by a drop in the circulating parasites
156 with complete recovery between day 20 and day 23 ($P < 0.05$) (Fig. 1a). Hematocrit percentage
157 was also compared between the two groups (Fig. 1b). B6 mice became severely anemic and died
158 probably from anemia. However, in DBA/2 mice there was an obvious response to anemia at the

159 peak that rapidly returned back to the control level during recovery ($P < 0.05$).

160

Fig. 1



161

162 **Fig. 1.** (a) Kinetics of parasitemia in B6 and DBA/2 mice during infection with lethal strain *P.*

163 *yoelii*. (b) Hematocrit percentages during malarial infection showing severe anemia at the severe

164 phase of B6 and the peak phase of DBA/2 mice ($n=4$ for each strain and each time-point).

165

166

167 3.2 Time kinetics of the number of lymphocytes in the liver and spleen during malarial 168 infection

169 The major organs where lymphocytes expanded during malarial infection were the liver and
170 spleen. The difference in the number of lymphocytes in the liver and spleen between control B6
171 mice and DBA/2 mice was compared (Fig. 2). Severe lymphocytosis was detected in the liver
172 and spleen of both B6 mice and DBA/2 mice after malarial infection. In DBA/2 mice, the
173 numbers of lymphocytes in the liver and spleen increased obviously towards day 20 after
174 infection ($P < 0.05$). As shown in Fig. 2, the acute phase appeared on day 3 in all mice, but the
175 severe phase was on day 7 in B6 mice and the recovery phase occurred on day 20 in DBA/2
176 mice.

Fig. 2

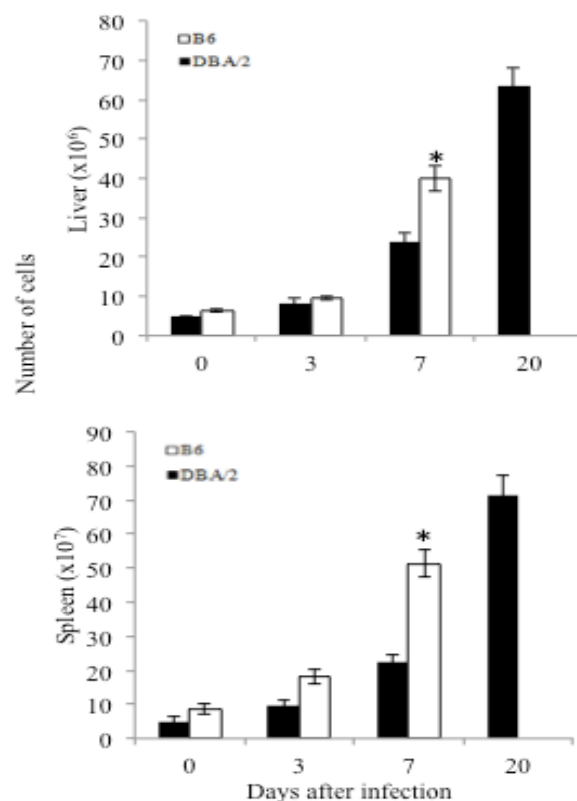


Fig. 2. Number of lymphocytes yielded by the liver and spleen in both B6 and DBA/2 mice during malarial infection. The acute phase started on day 3 in all mice, and the severe phase occurred on day 7 in B6 mice and the recovery phase on day 20 in DBA/2 mice (n=4 for each strain and each time-point). * P < 0.05

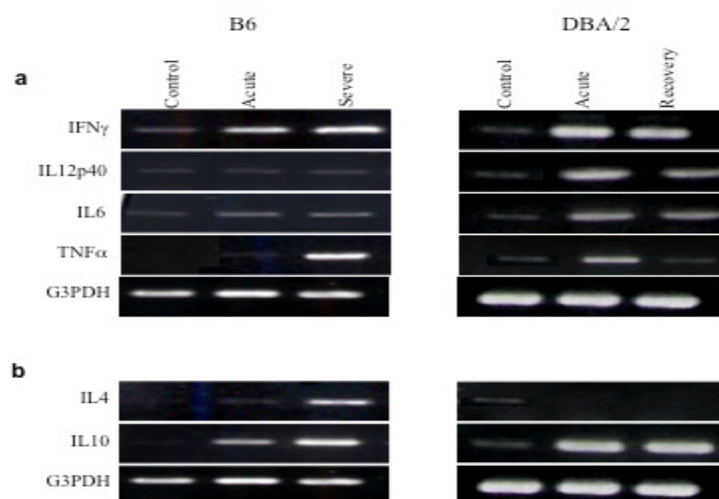
3.3 Cytokine production by RT-PCR

The expression of cytokine mRNA of B6 mice and DBA/2 upon challenge with infection was evaluated. Inflammatory cytokines mRNA (i.e., IFN γ , IL-12p40, IL-6, and TNF- α) and anti-inflammatory cytokines (i.e., IL-4 and IL-10) mRNA were detected in the liver tissue by RT-PCR (Fig. 3). The production of IFN- γ mRNA increased in both B6 and DBA/2 mice with

188 higher expression in the acute phase of DBA/2 mice. Significant levels of IL-12p40 mRNA were
 189 not appeared in B6 mice compared with the higher expression in DBA/2 mice. The expression
 190 level of IL-6 mRNA was quite similar to that of IL-12 in both B6 and DBA/2 mice. The
 191 production of TNF- α sharply increased in the severe phase in B6 mice. In contrast, the mRNA
 192 levels of TNF- α clearly dominated during the recovery phase of DBA/2 mice (Fig. 3a). The
 193 mRNA expression profile for IL-4 and IL-10 was determined as representatives of anti-
 194 inflammatory response (Fig. 3b). The production of IL-4 mRNA increased during the severe
 195 phase of B6 mice. On the other hand, no significant induction of IL-4 was detected in DBA/2
 196 mice. IL-10 mRNA showed more expression in DBA/2 mice than B6 mice.

197

Fig. 3



198 **Fig. 3.** Detection of cytokines mRNA from the liver tissues of B6 and DBA/2 mice during
 199

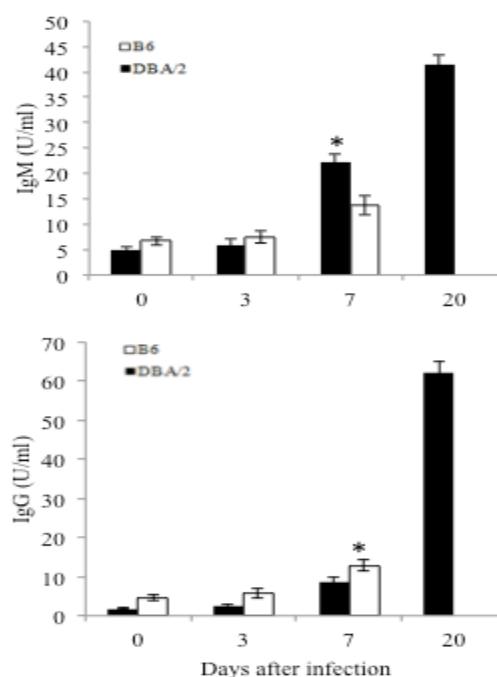
200 malarial infection. Expression of some inflammatory cytokines (a) and anti-inflammatory
 201 cytokine (b) at the indicated points of time. The integrity of RNA was confirmed by the
 202 detection of G3PDH. Representative results of four experiments are depicted.

203 3.4 Autoantibody production during malarial infection

204 The levels of both IgM-type and IgG-type autoantibodies against denatured DNA were measured
 205 (Fig. 4). In B6 mice both IgM-type and IgG-type autoantibodies showed little increase from the
 206 acute phase till the severe phase. In sharp contrast, in DBA/2 mice both IgM-type and IgG-type
 207 autoantibodies showed maximum increase during recovery ($P < 0.05$).

208

Fig. 4



209

Fig. 4. Autoantibody production in B6 and DBA/2 mice after malarial infection. Both IgG and IgM types of autoantibodies against denatured DNA were detected by ELISA. Sera of *lpr* mice were used as a positive control (adjusted as 100 U/mL). * $P < 0.05$

3.5 Liver injury induced by malarial infection

Malaria infection provokes liver inflammation and injury. In this context, we assessed the serum level of GPT (Fig. 5). A prominent elevation in GPT level was seen in B6 mice after malarial infection. However, such elevation was less prominent in DBA/2 mice ($P < 0.05$).

Fig. 5

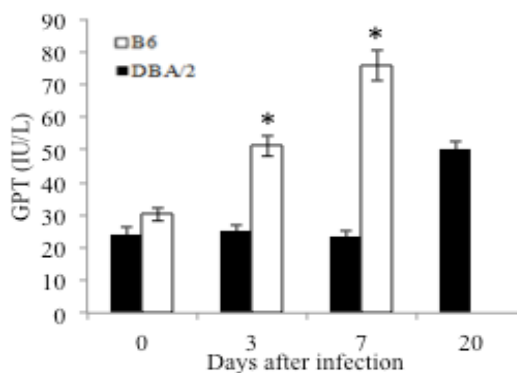
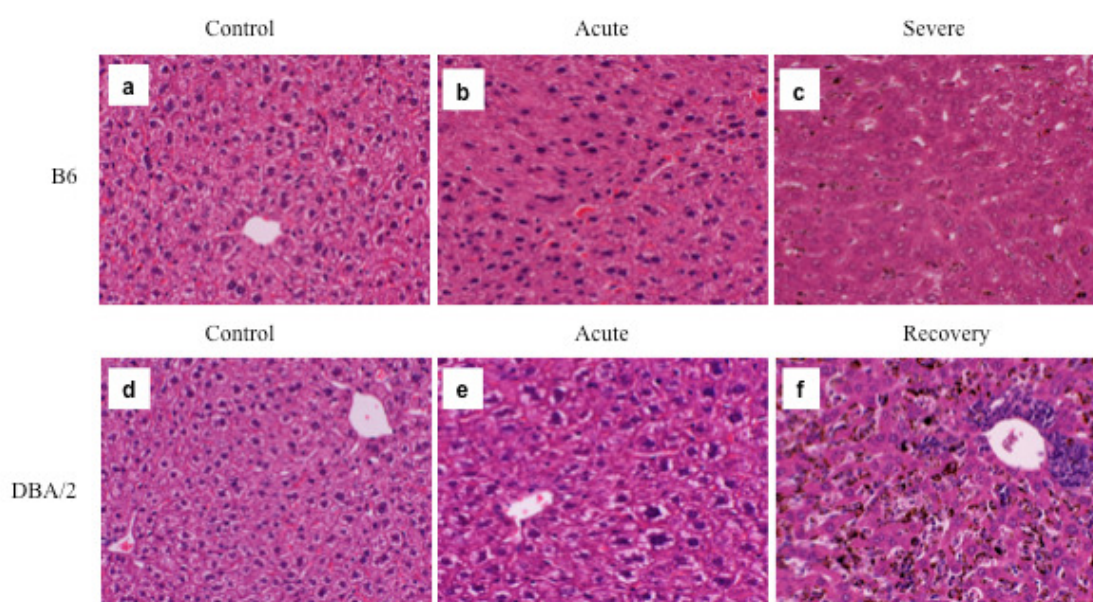


Fig. 5. Assays for glutamic pyruvic transaminase (GPT). Sera were obtained at the indicated points of time from B6 and DBA/2 mice. * $P < 0.05$

220 3.6 Histology of the liver after malaria infection

221 To determine the magnitude of inflammation during malarial infection, the histology of the livers
 222 was examined in both B6 and DBA/2 mice (Fig. 6). Histology of liver explained the above-
 223 mentioned situation regarding the elevation of GPT in sera, which showed severe liver damage
 224 in both B6 mice (Fig. 6 a, b, c) and DBA/2 mice (Fig. 6 d, e, f) with clusters of lymphocytes and
 225 hemozoin pigment more obvious at the recovery phase of DBA/2 mice.

Fig. 6



226 **Fig. 6.** Liver cell damage and accumulation of lymphocyte in the liver of B6 and DBA/2 mice
 227 after malarial infection. (a, b and c) infected B6 mice; (d, e and f) infected DBA/2 mice.
 228 Histology of the liver stained with H&E (×20).

4. DISCUSSION

The target of immunological research on malaria over the past three decades has been for vaccine development. The main bulk of such research focused on adaptive immune responses, yet the innate response to malaria has received little attention. However, a series of studies have demonstrated the protective role of innate immune mechanisms, which increases the expectation to reach a suitable and effective malaria vaccine [11, 18, 25]. Experimental murine malaria explained and updated the understanding of how *Plasmodium* parasites interact with the host immune responses [30]. Previous studies have illustrated that the resistant DBA/2 mice infected with *P. yoelii* (lethal and non lethal strain) were associated with different outcomes from those found with B6 mice infected with the same strains [11, 12, 24].

The present study was conducted for further investigation of the immunological basis of the virulence strain of the malaria parasite (*P. yoelii* 17XL), wherein the mRNA expression profile for some inflammatory and anti-inflammatory cytokines was detected in two different strains of mice (B6 and DBA/2). This study demonstrated that DBA/2 mice reduced parasitemia through rapid induction of cytokines such as Th1- and Th2-type cytokines that were correlated with the stages of infection. In this experiment it was observed that the level of inflammatory cytokines such as IFN- γ was higher in DBA/2 mice than in B6 mice, something which enabled the DBA/2 mice to control the replication of parasites, which might be useful during the acute stage of infection. This observation agrees with earlier studies where it was shown that the administration of recombinant IFN- γ increased the survival of susceptible mice infected with LPY 17X [31]. Our data demonstrated that the timing of TNF- α production was beneficial, as its level increased in the acute stage in the resistant DBA/2 mice while its production was late in the severe phase of

252 B6 mice. In other words, early production of TNF- α is protective in DBA/2 while late
 253 production may be serious in severe phase of B6 mice. This observation is consistent with
 254 another work suggesting that early TNF- α responses have been implicated in protective
 255 immunity against *P. chabaudi* AS in resistant mouse strains [32]. The elevated levels of IFN- γ
 256 and TNF- α are particularly remarkable since they are involved in parasite clearance and
 257 preservation of erythropoiesis [33]. The present work focused on the protective response of
 258 another inflammatory cytokine (IL-12), which was more highly produced in DBA/2 mice than in
 259 B6 mice. The macrophage activation may be the first response to occur after infection, giving
 260 rise to the production of IL-12 and TNF- α [34], both of which stimulate NK and Th1 cells to
 261 produce IFN- γ [35]. These results are in line with a previous work where it was shown that IL12
 262 and IFN γ played a vital role in keeping *P. falciparum* from causing malignant malaria [36]
 263 However, on the contrary to the results of the present study, Waki et al. [37] have shown that IL-
 264 12 is implicated in pathogenesis instead of providing protection during lethal *P. berghei* NK65
 265 infection which might be due to the difference in the quantity produced, the site of its production,
 266 the time during which its production is sustained, and the presence of other molecules regulating
 267 its production. The present data showed a significant correlation between IL-12 and IL-6 as their
 268 production was elevated in all phases of DBA2 mice, unlike their expression in B6 mice, which
 269 suggested their coordinated production. Moreover, a strong correlation was documented in a
 270 recent study between IL-6 and TNF- α [38]. These pro-inflammatory cytokines act
 271 synergistically; thus, the outcome of these early cytokine responses could be critical in activating
 272 the effector mechanisms necessary for the resolution of parasitemia. Our results are consistent
 273 with earlier observations, which illustrated that an early pro-inflammatory cytokine mRNA
 274 expression leads to successful resolution of malaria infection in laboratory-bred AKR mice

275 during nonlethal *P. vinckei* arteether resistant (PvAR) infection [39].

276 Results of other studies support the idea that pro-inflammatory cytokines such as IFN γ , IL-12
277 and TNF α are accompanied with the severity of malaria infection including cerebral malaria [40,
278 41]. Coban et al. [42] examined the role of myeloid differentiation primary response gene 88
279 (MyD88)-dependent Toll-like receptor (TLR) on the systemic productions of IFN γ , IL-12 and
280 TNF α cytokines. The results showed that the serum levels of such cytokines and hemozoin
281 accumulation were significantly reduced in MyD88-deficient mice infected with *Plasmodium*
282 *berghei* ANKA.

283 The PCR analysis of the mRNA expression of anti-inflammatory cytokines (IL-4 and IL-10)
284 during malaria infection in DBA/2 mice showed that the expression of IL-10 was found to be
285 high in sharp contrast to that of IL-4, which was very low. This observation suggested that IL-10
286 may have the upper hand over IL-4 in controlling the high level of inflammatory cytokines in the
287 early stage and continued their effect till recovery to minimize pathology. Therefore, anti-
288 inflammatory cytokines are not equally important for the suppression of the inflammatory
289 response that leads to successful resolution of blood stage malaria. Our results are consistent
290 with earlier observations that showed that the pattern of Th2 cytokines during immune responses
291 is to limit the outcome of an exaggerated pro-inflammatory response and to counterbalance the
292 production of Th1 cytokines [43]. Previous research has shown that the activation of regulatory
293 T cells (Tregs) was accompanied by high levels of IL-10 [44, 45]. This speculation is based on
294 the crucial roles of Tregs in regulating Th1/Th2 responses and in the successful shift of Th1 to
295 Th2 cells [46, 47]. Therefore, our study indicated that we could use IL-10 at the appropriate time
296 to promote a successful switch between th1 and th2 responses in murine malaria.

In parallel with the production of cytokines during malaria infection, another important finding in this study was the detection of IgM and IgG type autoantibody against denatured DNA, which continued to increase in the sera of DBA/2 mice especially during the recovery phase. This immunologic response might be a compensatory function in DBA/2 mice that are known to be deficient in CD8⁺ cytotoxic T-cell function [11, 48]. The activation of autoantibody-producing B-1 cells occurred due to the nature of *Plasmodium* as an intracellular pathogen, which resembled those seen in autoimmune diseases [49- 51].

The final step in this experiment was to check the histology of the liver, as it was previously reported that the liver was the organ most seriously affected by inflammation after malarial infection [52]. Our observations demonstrated that hepatic damage occurred in both types of mice; however, the GPT level in sera was higher in susceptible B6 mice than in DBA/2 mice.

5. CONCLUSIONS

Taken together, this study has given valuable evidence about the characteristic pattern of cellular cytokines mRNA expression in susceptible B6 mice and protected DBA/2 mice infected with virulent strain *P. yoelii*. It can be deduced that the balance between the cytokines and the timing of their production is crucial. Further research is needed to explore the validation of the role of IL-6 and IL-10 agonists and their mode of action.

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