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

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Authors' contributions

This work was carried out in collaboration between both authors. Author HYB wrote the protocol, designed the study, managed the experimental part and data collection. Author HYB wrote the first draft and finalized manuscript. Author CTM helped in the experimental part, confirming the results and did the statistical analysis. Authors HYB and CTM shared in analysis of the data and revise the manuscript. Both authors read and approved the final manuscript.

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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 (IFNγ), Interleukins (IL)-12, IL-6, and tumor necrosis factor-α (TNFα)] 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 TNFα 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

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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 give valuable evidence about the characteristic pattern of cellular cytokines mRNA expression and their role in controlling malaria in two different strains of mice. Hence presenting 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 IFNγ, IL-12, IL-6, TNFα 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: Thl 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 IFNγ) 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℃ 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 NH_4Cl, 10 mM $KHCO_3$, 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 IFNγ, IL-12P40, IL-6, TNFα, 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 µg 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 $MgCl_2$, 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℃ for 1 min, annealing at 60℃ for 1 min and elongation at 72℃ 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-α (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℃ 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).

2.7 Histology

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

2.8 Statistical Analysis

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

3. RESULTS

3.1 Survival Conditions of DBA/2 Mice Infected with Malaria

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

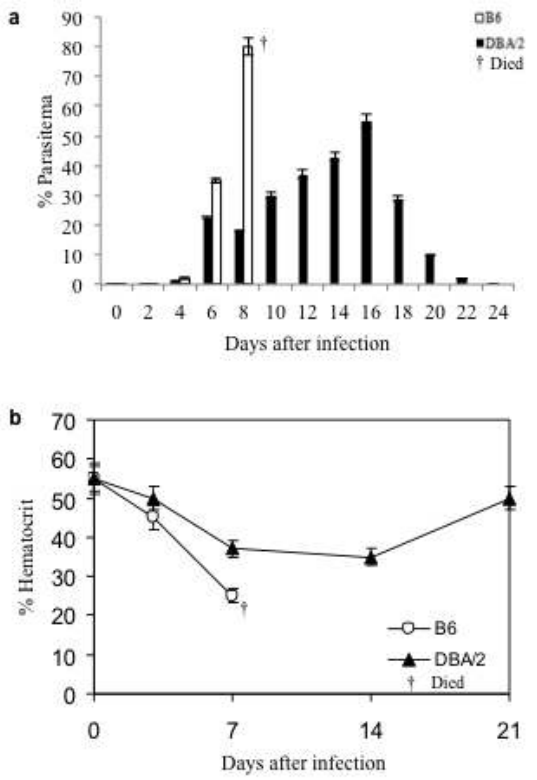

Fig. 1. (a) Kinetics of parasitemia in B6 and DBA/2 mice during infection with lethal strain P. yoelii. (b) Hematocrit percentages during malarial infection showing severe anemia at the severe phase of B6 and the peak phase of DBA/2 mice (n=4 for each strain and each time-point)

3.2 Time Kinetics of the Number of Lymphocytes in the Liver and Spleen during Malarial Infection

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

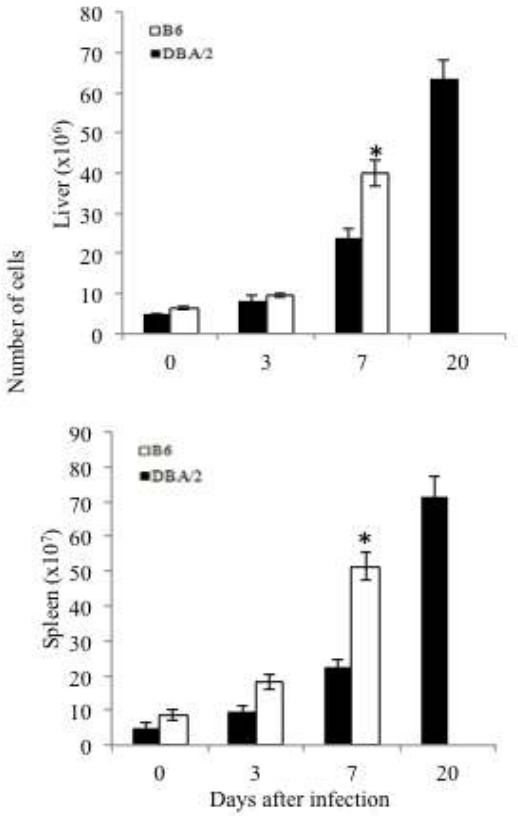

Fig. 2. Number of lymphocytes yielded by the liver and spleen in both B6 and DBA/2 mice during malarial infection. The acute phase started on day 3 in all mice, and the severe phase occurred on day 7 in B6 mice and the recovery phase on day 20 in DBA/2 mice (n=4 for each strain and each time-point)

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

3.4 Autoantibody Production during Malarial Infection

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

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 ($P < 0.05$).

3.6 Histology of the Liver after Malaria Infection

To determine the magnitude of inflammation during malarial infection, the histology of the livers was examined in both B6 and DBA/2 mice (Fig. 6). Histology of liver explained the above-mentioned situation regarding the elevation of GPT in sera, which showed severe liver damage in both B6 mice (Fig. 6a, b, c) and DBA/2 mice (Fig. 6d, e, f) with clusters of lymphocytes (indicated by arrow) and hemozoin pigment (double arrow) more obvious at the recovery phase of DBA/2 mice. A photomicrographic section of the liver in (Fig. 6b, c, e, f) revealed areas of degeneration in the cytoplasm of liver cells in the form of areas of vacuolations, the nuclei showed variability in size but most of the cells still having euchromatic visible nucleolie.

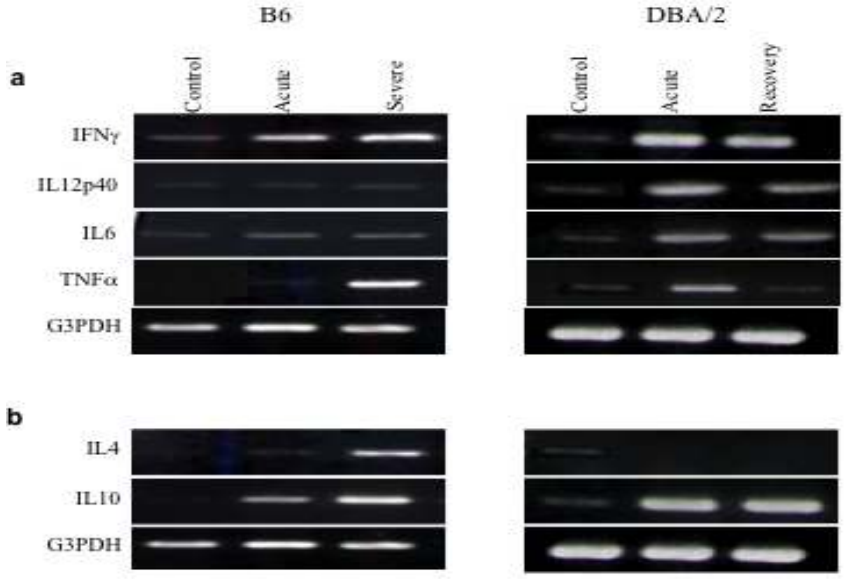

Fig. 3. Detection of cytokines mRNA from the liver tissues of B6 and DBA/2 mice during malarial infection. Expression of some inflammatory cytokines (a) and anti-inflammatory cytokine (b) at the indicated points of time. The integrity of RNA was confirmed by the detection of G3PDH. Representative results of four experiments are depicted

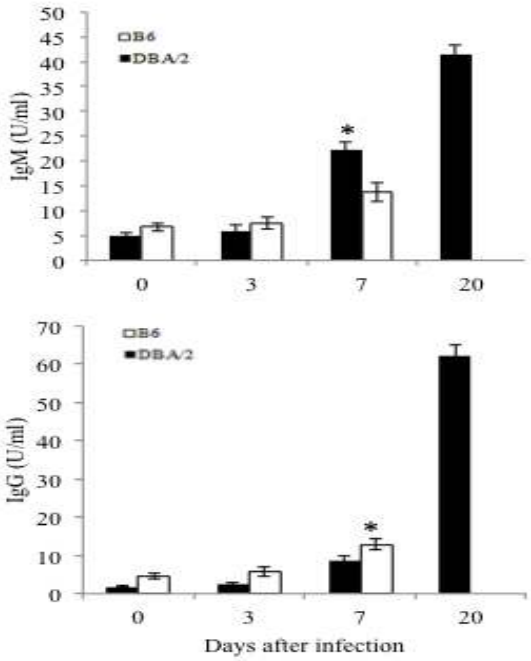

Fig. 4. Autoantibody production in B6 and DBA/2 mice after malarial infection. Both IgG and IgM types of autoantibodies against denatured DNA were detected by ELISA. Sera of *lpr* mice were used as a positive control (adjusted as 100 U/mL)

** P < 0.05*

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 B6 mice. In other words, early production of TNF-α is protective in DBA/2 while late production may be serious in severe phase of B6 mice. This observation is consistent with another work suggesting that early TNF-α responses have been implicated in protective immunity against *P. chabaudi* AS in resistant mouse strains [32]. The elevated levels of IFN-γ and TNF-α are particularly remarkable since they are involved in parasite clearance and preservation of erythropoiesis [33]. The present work focused on the protective response of another inflammatory cytokine (IL-12), which was more highly produced in DBA/2 mice than in B6 mice. The macrophage activation may be the first response to occur after infection, giving rise to the production of IL-12 and TNF-α [34], both of which stimulate NK and Th1 cells to produce IFN-α [35]. These results are in line with a previous work where it was shown that IL12 and IFNα played a vital role in keeping *P. falciparum* from causing malignant malaria [36] However, on the contrary to the results of the present study, Waki et al. [37] have shown that IL-12 is implicated in pathogenesis instead of providing protection during lethal *P. berghei* NK65 infection which might be due to the difference in the quantity produced, the site of its production, the time during which its production is sustained, and the presence of other molecules regulating its production. The present data showed a significant correlation between IL-12 and IL-6 as their production was elevated in all phases of DBA2 mice, unlike their expression in B6 mice, which suggested their coordinated production. Moreover, a strong correlation was documented in a recent study between IL-6 and TNF-α [38]. These pro-inflammatory cytokines act synergistically; thus, the outcome of these early cytokine responses could be critical in activating the effector mechanisms necessary for the resolution of parasitemia. Our results are consistent with earlier observations, which illustrated that an early pro-inflammatory cytokine mRNA expression leads to successful resolution of malaria infection in laboratory-bred AKR mice during nonlethal *P. vinckei* arteether resistant (PvAR) infection [39].

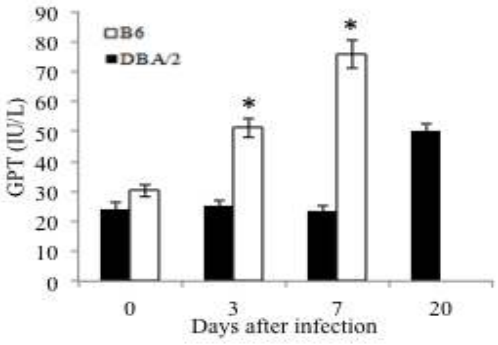

Fig. 5. Assays for glutamic pyruvic transaminase (GPT). Sera were obtained at the indicated points of time from B6 and DBA/2 mice

** P < 0.05*

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

The PCR analysis of the mRNA expression of anti-inflammatory cytokines (IL-4 and IL-10) during malaria infection in DBA/2 mice showed that the expression of IL-10 was found to be high in sharp contrast to that of IL-4, which was very low. This observation suggested that IL-10 may have the upper hand over IL-4 in controlling the high level of inflammatory cytokines in the early stage and continued their effect till recovery to minimize pathology. Therefore, anti-inflammatory cytokines are not equally important for the suppression of the inflammatory response that leads to successful resolution of blood stage malaria. Our results are consistent with earlier observations that showed that the pattern of Th2 cytokines during immune responses is to limit the outcome of an exaggerated pro-inflammatory

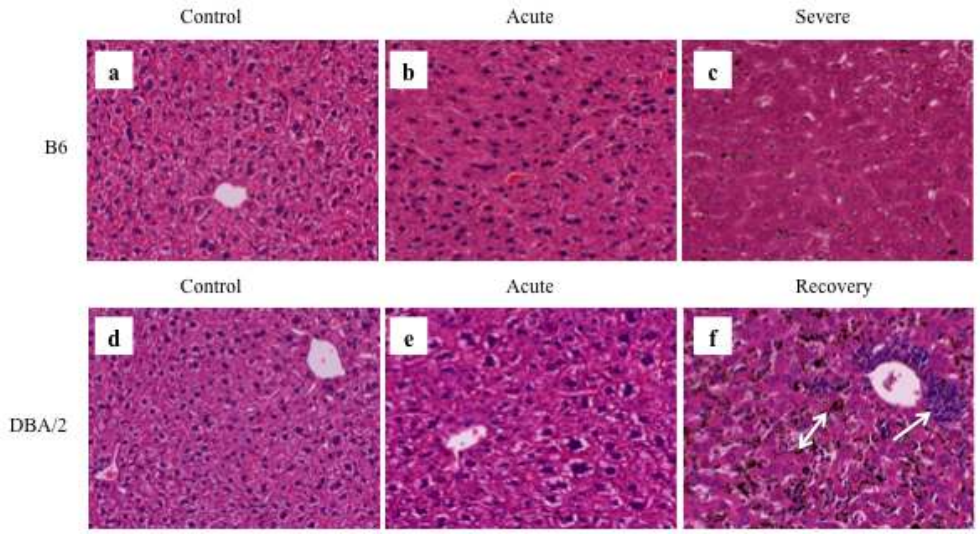

Fig. 6. Liver cell damage and accumulation of lymphocyte in the liver of B6 and DBA/2 mice after malarial infection. (a, b and c) infected B6 mice; (d, e and f) infected DBA/2 mice. Histology of the liver stained with H&E (×20)

response and to counterbalance the production of Th1 cytokines [43]. Previous research has shown that the activation of regulatory T cells (Tregs) was accompanied by high levels of IL-10 [44,45]. This speculation is based on the crucial roles of Tregs in regulating Th1/Th2 responses and in the successful shift of Th1 to Th2 cells [46,47]. Therefore, our study indicated that we could use IL-10 at the appropriate time 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.

CONSENT

It is not applicable.

ETHICAL APPROVAL

The experiments were done in the animal house, Department of Immunology, School of Medicine, Niigata University, Niigata, Japan. Animal house ethical committee approved them. Animal handling protocols meet the standard international guidelines by the National Institutes of Health guide for the care and use of Laboratory animals. All mice were maintained in pathogen-free insulators. Human endpoints to reduce pain or distress in mice were used via

euthanasia. Mice were sacrificed using CO_2 after which the cervical dislocation method was used.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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