MODULATING EFFECTS OF IL-4, IL-10 AND IL-13 ON THE COURSE OF *PLASMODIUM BERGHEI* MALARIA INFECTION IN MICE

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Abstract

Background: Inflammation is a crucial process driving pathogenesis in malaria infection. The devastating effects of malaria infection has always been associated with severe inflammation whilst protective effect is linked to provocation of anti-inflammation responses. IL-4, IL-10 and IL-13 are well-established anti-inflammatory cytokines with their functional roles during malaria infection remain elusive. Therefore, this study was undertaken to study the effects of modulating IL-10, IL-4 and IL-13 on the course of malaria infection in *Plasmodium berghei* ANKA (PbA)-infected murine model.

Methods: Male ICR mice were randomly assigned into 5 different groupings and were infected intraperitoneal with 0.2 mL of 2 x 10^7 pRBCs containing *P. berghei* ANKA (PbA). Malaria-infected mice were treated with recombinant mouse IL-4 (rmIL-4), recombinant mouse IL-10 (rmIL-10) and recombinant mouse IL-13 (rmIL-13) for 4 consecutive days after the establishment of the infection. The survival and parasitemia levels of malarial mice and malarial mice under different treatments were monitored. Major affected organs (kidneys, lungs, brain, liver and spleen) were subjected to histopathological analysis at day-5 post infection.

Results: Our findings revealed that the overall lifespan of malarial mice treated with recombinant mouse rmIL-10, rmIL-4 and rmIL-13 were prolonged, accompanied with significant reduction in malaria parasitemia levels, in particular in malarial mice receiving recombinant rmIL-10 and rmIL-13. Histopathological conditions of kidneys, lungs, brain, liver and spleen treated with recombinant mouse rmIL-10, rmIL-4 and rmIL-13 were also improved. Sequestration of parasitized red blood cells (pRBCs) and inflammation seen in major affected organs were alleviated.

Conclusion: Despite some limitations, this preliminary study demonstrated the promising therapeutic effects of IL-10 and IL-13 as adjuvant therapies in reducing severe pathological manifestations triggered by inflammation during malaria infection.

Keywords: IL-4, IL-10, IL-13, Malaria, *Plasmodium berghei,* Histopathology

Introduction

Malaria remains one of the major vector-borne diseases that gathered attention globally. Malaria is caused by *Plasmodium* parasites and is being transmitted primarily to humans via bites of *Anopheles* mosquito. Currently, there are five *Plasmodium* species infecting humans namely *Plasmodium vivax*, *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium ovale*, and the recently discovered simian *Plasmodium knowlesi* (1, 2). Once malaria parasites have successfully invaded the human hosts, they started to grow and multiply in the liver as well as in the red blood cells where most of the clinical symptoms such as chills, headaches and fever appeared. However, these clinical symptoms are non-specifics which might lead to differential clinical diagnosis consisting of dengue, typhoid fever, viral hepatitis and other infections (3). To date, most of the current anti-malarial strategies have been targeted on *Plasmodium* parasites, however malaria infection remains to be endemic due to development of drug resistance towards anti-malarial drugs and rapid increment of mosquito insecticide resistance. Many attempts have been made to facilitate the development

of effective malaria treatments and vaccines. Further, incomplete understanding of protective immunity and its core induction process has hindered the effectiveness of malaria control measures (4, 5).

Over the last three decades, there has been notable increased in the amount of laboratory-based studies on murine models of malaria infection. Four rodent malaria species comprising of *Plasmodium chabaudi*, *Plasmodium berghei*, *Plasmodium yoelii*, and *Plasmodium vinckei* are the most commonly malaria parasites being used in different host-parasite combination to investigate the mammalian immune response towards malaria. For instance, *P. chabaudi* which shares several similarities with *P. falciparum* during asexual stage is suitable for the studies relating to the development of blood stage vaccine. *P. berghei* ANKA (PbA) infection in susceptible (CBA or C57BL/6 mice) or resistant (BALB/c) models are, however, the most extensively studied murine model of cerebral malaria (CM). It developed cerebral features and parasite sequestration in microcirculation of major organs that strikingly resembled those in human CM. *P. yoelii* 17XNL is extensively studied in multiple mice strains in order to understand malaria pathogenesis and its protection mechanisms whilst lethal *P. yoelii* YM strain that attacked both reticulocytes and mature RBC is often being employed for vaccine candidate testing (6). Although experimental murine model of malaria infection could be an informative platform for detailed examination of pathophysiological conditions caused by malaria parasites, but it is essential to ensure that the disease pattern observed in humans is mirrored as closely by the experimental models used (7).

Anti-inflammatory cytokines are important regulators in malaria infection. These cytokines could either suppress the activities of pro-inflammatory cytokines or inhibited the production of specific immune response in host (8). Anti-inflammatory cytokines such as interleukin 4 (IL-4), interleukin 10 (IL-10), transforming growth factor beta (TGF-β) and interleukin 13 (IL-13) confer protection in murine malaria during the late acute phase of malaria infection (9). For instance, IL-4 inhibits the expression of pro-inflammatory cytokines including tumour necrosis factor alpha (TNF-α) where its high expression levels have been associated to exacerbation of the severity of malaria infection (9). Additionally, neutralization of IL-4 demonstrated by several studies using rodent malaria model increases the malaria parasitemia levels along with reduced production of nitric oxide, suggesting the involvement of IL-4 in severe malaria pathogenesis (10, 11). On the other side, IL-10 is a potent deactivator of T-helper (Th_1) cytokine. It regulates host immune responses via inhibition of TNF-α and interferon gamma (IFN-γ) productions, as seen in malaria and other community-acquired infections. Dysregulation of IL-10 secretions had been found to intensify the severity of malaria infection (12-14). IL-13 is an anti-inflammatory mediator that modulates host immune responses of monocytes and B-cells (15). Previous study had demonstrated the association of IL-13 with reduced expression of surface adhesion molecules on both monocytes and macrophages during malaria infection (16). Also, IL-13 has emerged as a negative marker for anemia in pediatric malaria patients (17).

Albeit the well-documented pathogenesis studies in malaria infection, there is still a large gap to be filled in understanding the host immune responses during malaria infection. Cytokines, particularly anti-inflammatory cytokines might be one of the potential targets for immunotherapeutic strategy in malaria. Therefore, this study attempted to investigate the modulating effects of IL-4, IL-10 and IL-13 cytokines on the course of malaria infection in *P. berghei* ANKA infection murine model (18), with an ultimate aim to determine whether these anti-inflammatory cytokines can ameliorate the immunopathological events caused by malaria parasite.

Materials and Methods

Animals and malaria infection

P. berghei ANKA (PbA) parasite obtained from Institute for Medical Research, Malaysia was used throughout the study. A total of 65 male ICR mice (5-6 weeks old) weighed between 18-25 g were used in this study. The animals were kept under mosquito-netted cage at animal house, Faculty of Medicine and Health Science, Universiti Putra Malaysia with food and water supply *ad libitum*. To establish malaria infection, ICR mice were infected intraperitoneal (i.p) with 0.2 mL of 2 x 10^7 parasitized red blood cells (pRBCs) obtained from a homologue donor mouse pre-infected with *P. berghei* ANKA (PbA). Control group (uninfected mice), however, received equal volume and dilution of normal red blood cells (RBCs) obtained from an uninfected donor mouse. All the experimental procedures used in the study followed regulations and guidelines approved by Animal Care and Use Committee of University Putra Malaysia (Approval No: UPM/FPSK/PADS/BR-UUH/00307).

Preparation of recombinant mouse IL-4 (rmIL-4), recombinant mouse IL-10 (rmIL-10) and recombinant mouse IL-13 (rmIL-13)

Recombinant mouse IL-4, IL-10 and IL-13 used in the study were purchased from R&D Systems, (USA). rmIL-4 was lyophilized from 84.7 µL of a 0.2 µm filtered phosphate-buffered solution (PBS) at pH 7.4 and was then reconstituted by adding 5 mL of sterile PBS. Similarly, lyophilized rmIL-13 was diluted with 2.5 mL of sterile PBS at room temperature. On the other hand, rmIL-10 was reconstituted by adding 2.15 mL of sterile 0.85% saline solution (Ain Medicare, Malaysia). All the solutions were stored at -80°C prior to use. The therapeutic dosage for rmIL-4 (19, 20), rmIL-10 (21, 22) and rmIL-13 (23) used in this study was 1 µg/0.1 mL, respectively.

Modulating effects of rmIL-4, rmIL-10 and rmIL-13 on the survival and malaria parasitemia of malarial mice

A total of 40 ICR male mice were randomly assigned into 5 groups consisting of 8 mice (n=8). All malarial mice were infected intraperitoneal with pRBCs (2 x 10^7, 0.2 mL) obtained from homologue donor, which had been inoculated with frozen stock of *P. berghei* ANKA (PbA). Control mice received equivalent volume and dilution of normal RBCs through the same route of administration. Starting from day-1 of post infection, doses of recombinant mouse cytokines were administered intravenously to groups of mice for 4 consecutive days (D1, D2, D3, D4). The mortality rate of all groups of mice were recorded up to day-10 of post infection whilst the parasitemia levels were monitored up to day-6 post infection.

The grouping of animals with respective treatment were as below:

Group I : Control mice receiving 0.01M PBS (0.1 mL, i.v) (C+PBS)

Group II : Malarial mice receiving 0.01M PBS (0.1 mL, i.v) (M+PBS)

Group III : Malarial mice treated with rmIL-4 (1µg, 0.1 mL, i.v) (M+IL-4)

Group IV : Malarial mice treated with rmIL-10 (1µg, 0.1mL, i.v) (M+IL-10)

Group V : Malarial mice treated with rmIL-13 (1µg, 0.1mL, i.v) (M+IL-13)

Parasitemia measurement

Parasitemia levels in malaria infected mice and malarial mice treated with rmIL-4, rmIL-10 and rmIL-13 were measured by collecting a drop of blood from the tail of the mice onto the edge of microscopic slide to make a thin blood film. Leishman's stain was then used to stain the thin blood smear and viewed under light microscope (Nikon eclipse 80i, USA) at 1000 x magnification with immersion oil (Nikon, USA). About 5 microscopic fields with an approximate 200 cells each were counted. The parasitemia was calculated as the total red blood cells that contain Leishman's positive bodies. The calculation formula for the estimation of parasitemia level in the mice was:

$$\frac{\text{Total number of Leishmain positive cell}}{\text{Total number of normal RBC}} \text{ X } 100\%$$

Effect of rmIL-4, rmIL-10 and rmIL-13 on the histopathological changes of malarial mice

A total of 25 ICR male mice were randomly divided into 5 groups consisting of 5 mice (n=5) and kept under mosquito-netted cage.

The grouping of animals with each respective treatment were as below:

Group I : Control mice receiving 0.01M PBS (0.1 mL, i.v) (C+PBS)

Group II : Malarial mice receiving 0.01M PBS (0.1 mL, i.v) (M+PBS)

Group III : Malarial mice treated with rmIL-4 (1µg, 0.1 mL, i.v) (M+IL-4)

Group IV : Malarial mice treated with rmIL-10 (1µg, 0.1mL, i.v) (M+IL-10)

Group V : Malarial mice treated with rmIL-13 (1µg, 0.1mL, i.v) (M+IL-13)

Recombinant mouse cytokines (rmIL-4, rmIL-10 and rmIL-13) were administered intravenously to groups of mice for 4 consecutive days (D1, D2, D3, D4) post infection. The 5 major organs including the brain, liver, spleen, kidney and lung were harvested from the mice for histopathological analysis at day-5 post inoculation as most of the mice succumbed to death at day-6 post infection. Organ perfusions were carried out intracardially using sterile PBS for 5 minutes before analysis. All major organs were fixed in 10% formalin and were subjected to tissue processing using automated tissue processor (Leica, Germany). Tissues were embedded in paraffin block and were sectioned into 5 µm thickness. The sectioned tissues were then stained with hematoxylin and eosin (H&E) with an autostainer (Leica, Germany). The histological changes of tissues between control mice, malarial mice and malarial mice treated with rmIL-4, rmIL-10 and rmIL-13 were assessed under light microscopy at 100 x and 400 x magnifications.

Statistical analysis

All statistical analyses were carried out using GraphPad Prism software packages version 5.0 (GraphPad Prism Inc., USA). Results were expressed as mean ± standard error of the means (SEM) for survival analysis. Kaplan-Meier estimator was used to compare the survival of malarial mice under different treatments with malarial mice treated with PBS. Parasitemia levels between different treatment groups (malarial mice treated with rmIL-4, rmIL-10 and rmIL-13) and malarial mice treated with PBS were compared using Kruskal-Wallis test followed by Dunn's post hoc analysis. Results were expressed as mean of percentage ± standard error of the means (SEM). A p-value less than 0.05 ($p<0.05$) was considered as statistically significant.

Results

Effects of treatment with rmIL-4, rmIL-10 and rmIL-13 on the parasitemia development of malarial mice

The parasitemia development of control and malarial mice under different treatment groups is shown in Figure 1. Parasitemia levels in malarial mice treated with PBS were consistently increased from day-1 until day-6 post infection, with a spike increase in malaria parasitemia was

observed from day-4 to day-5 post infection, from 38% to 62%, respectively. Further, a peak parasitemia of 74% was recorded in malarial mice at day-6 post infection. On the other side, significant increase in malaria parasitemia was observed in malarial group treated with rmIL-13 at day-3 and day-4 post infection compared to malarial group treated with PBS. In contrast, significant reduction in parasitemia development was recorded in malarial group treated with rmIL-10 at day-4, day-5 and day-6 post infections whilst significant inhibition on malaria parasitemia levels in malarial mice treated with rmIL-13 was found at day-5 and day-6 post infections compared to malarial mice treated with PBS. Significant reduction in malaria parasitemia levels in mice treated with rm-IL4 was not recorded throughout the study.

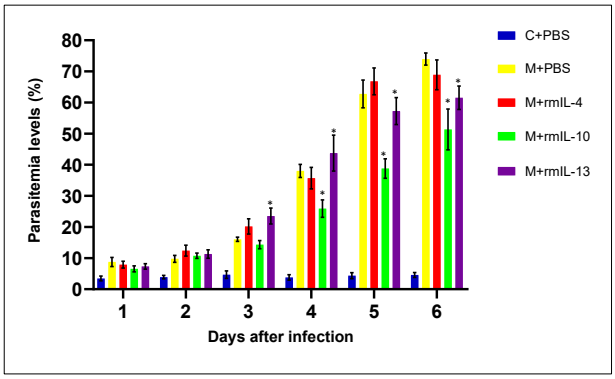


Figure 1: Parasitemia levels of control uninfected mice and malarial mice under different treatment groups. Parasitemia levels between different treatment groups were analyzed using Kruskal-Wallis test followed by Dunn's post hoc analysis. Results were expressed as mean of percentage ± standard error of the means (SEM). Keynote: * denotes $p<0.05$; C+PBS = Control + PBS solution; M+PBS = Malaria + PBS solution; M+rmIL-4 = Malaria + recombinant mouse IL-4; M+rmIL-10 = Malaria + recombinant mouse IL-10; M+rmIL-13 = Malaria + recombinant mouse IL-13

Effects of treatment with rmIL-4, rmIL-10 and rmIL-13 on the survival rate of malarial mice

The survival rates of control and malarial mice under different treatment groups is depicted in Figure 2. Uninfected control mice showed 100% survival rate throughout the study while PBS-treated malarial mice succumbed to death at day-7 post infection. The lifespan of malarial mice receiving recombinant mouse anti-inflammatory cytokines (rm-IL4, rmIL-10 and rm-IL13) were prolonged compared to malarial mice treated with PBS. Malarial mice from M+rmIL-13 group succumbed to death at day-9 post infection whilst the mortality rates of malarial mice from M+rmIL-4 and M+rmIL-10 groups reached 100% at day 10 post infection (Figure 2).

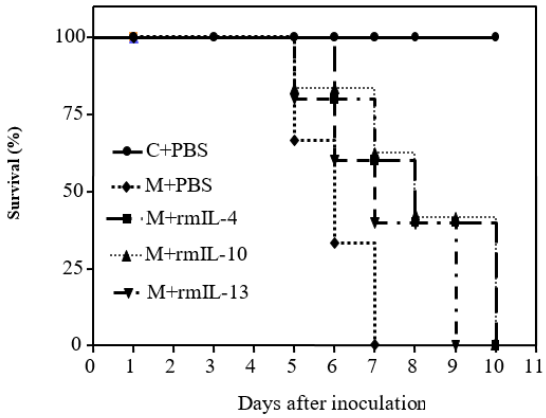
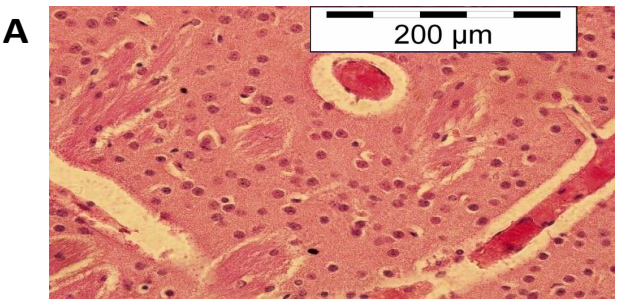


Figure 2: Percentage survival of Control and Malarial Mice Treated with rmIL-4, rmIL-10 and rmIL-13. Data were analyzed using Kaplan-Meier survival estimator. Keynote: C+PBS = Control + PBS solution; M+PBS = Malaria + PBS solution; M+rmIL-4 = Malaria + recombinant mouse IL-4; M+rmIL-10 = Malaria + recombinant mouse IL-10; M+rmIL-13 = Malaria + recombinant mouse IL-13

Effects of treatment with rmIL-4, rmIL-10 and rmIL-13 on the histopathological conditions of malarial mice

Brain

Severe cerebral pathology particularly sequestration of pRBCs in the cerebral vessels was observed in PBS-treated malarial mice (Figure 3B). Most of the neurons appeared to be pyknotic in association with hypercellularity, suggesting the occurrence of neurodegeneration (Figure 3B). The absence of pyknotic neurons in malarial mice treated with recombinant rmIL-4, rmIL-10 and rmIL-13 improvised the morphology of neurons (Figure 3C, 3D and 3E). However, the perivascular space of all malarial mice remains to be enlarged (Figure 3B). Sequestered pRBCs was not found accumulated in adjacent to the microvascular endothelial wall of malarial mice treated with rmIL-10, rmIL-4 and rmIL-13 (Figure 3C, 3D and 3E).



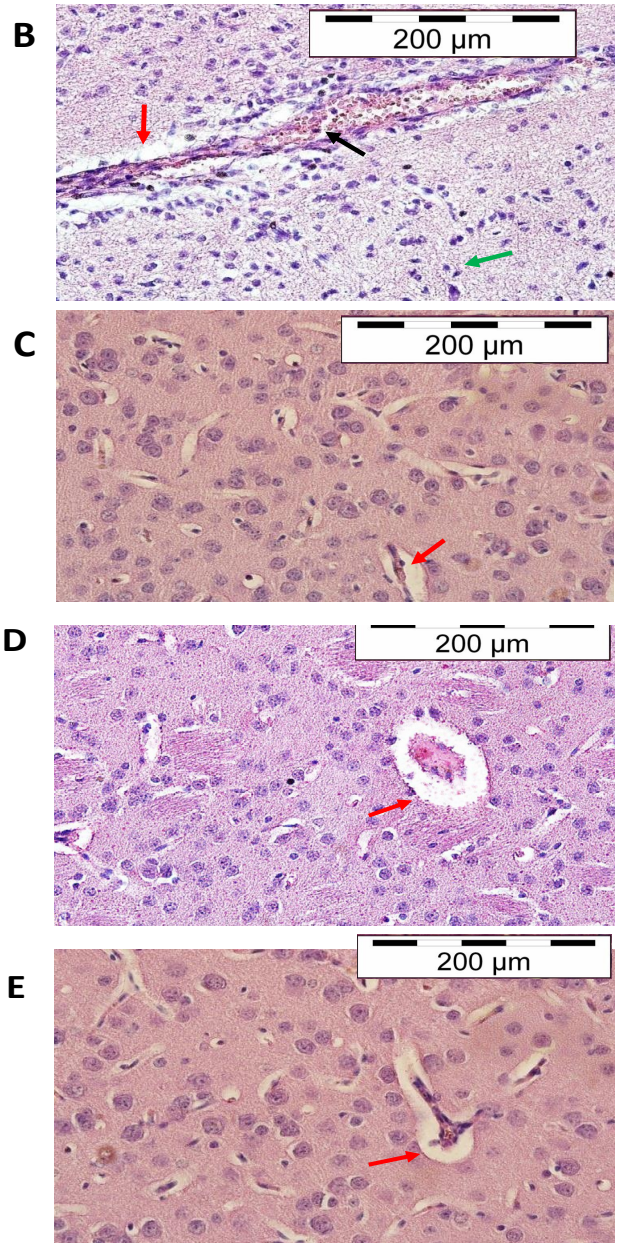
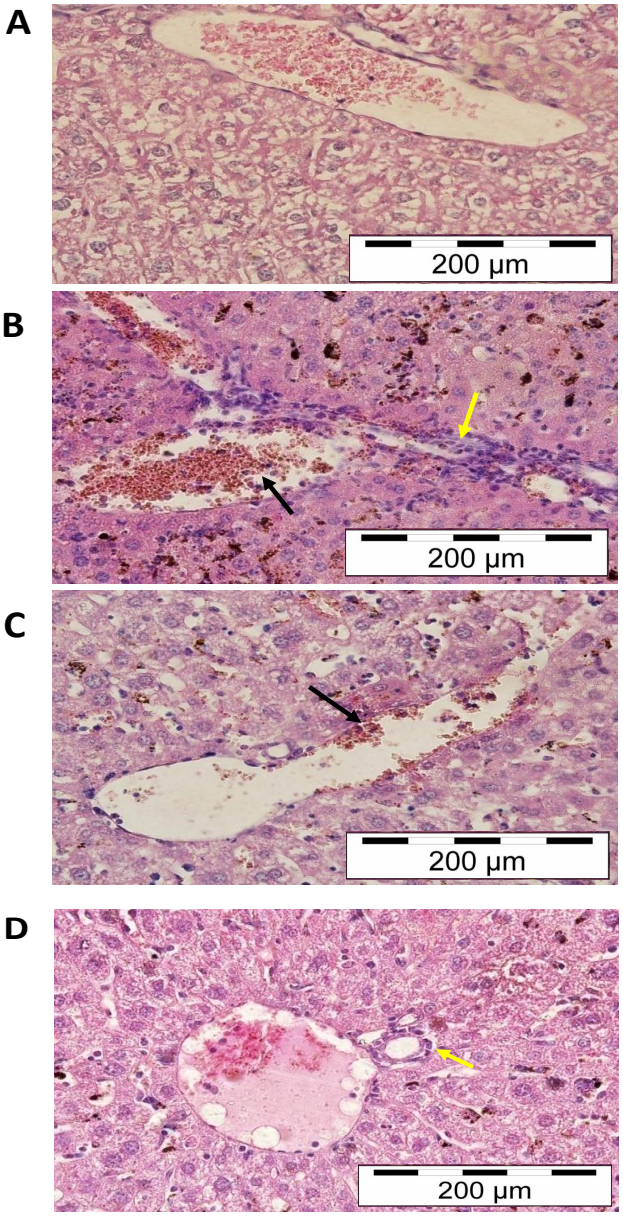


Figure 3: Light Micographs of Brain Tissue of PBS-treated Control Mice (A), PBS-treated- (B), rmIL-4-treated (C), rmIL-10-treated (D) and rmIL-13-treated (E) Malarial mice. Sequestration of pRBCs and macrophage engulfing elements (black arrow) were observed in microvessels in association with pyknotic neurons (green arrow) in brain tissue of PBS-treated malarial mice. Neuronal cells in malarial mice receiving anti-inflammatory cytokine related drugs appeared to be normal. Enlarged perivascular spaces (red arrow) were present in all malarial mice. H & E stain, x400

Liver

Acute liver injury was observed in PBS-treated malarial mice. The injury could be a consequence of pRBCs sequestration within the hepatic portal vein and inflammation, evident by severe lymphocytic infiltrations around the portal tract and perivascular areas (Figure 4B). Massive congestion of malarial pigments in hepatic cord accompanied with significant dilatation of sinusoids were observed (Figure 5B). Abundance of hypertrophied and hyperplasia Kupffer cells attributed to enlarged liver were observed in PBS-treated malarial mice (Figure 5B). These hepatic lesions were also found in rmIL-4, rmIL-10 and rmIL-13 treated malarial mice but to a lesser extent compared to PBS-treated malarial mice (Figure 4C, 4D and 4E). The appearance of hypertrophied Kupffer cells and dilated sinusoids were mitigated in liver tissues of malarial mice receiving these anti-inflammatory cytokines (Figure 5C, 5D and 5E). A reduction in pigment clumping with visibly smaller and more scattered appearance was observed in malarial mice treated with rmIL-4, rmIL-10 and rmIL-13 (Figure 5C, 5D and 5E).



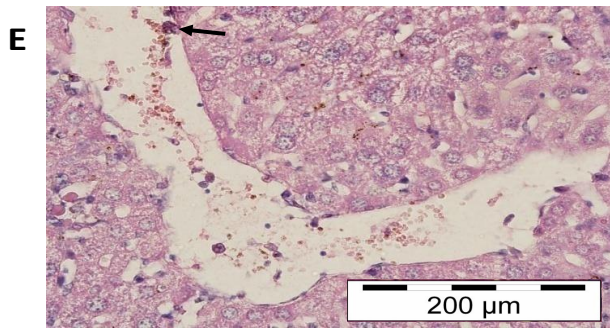
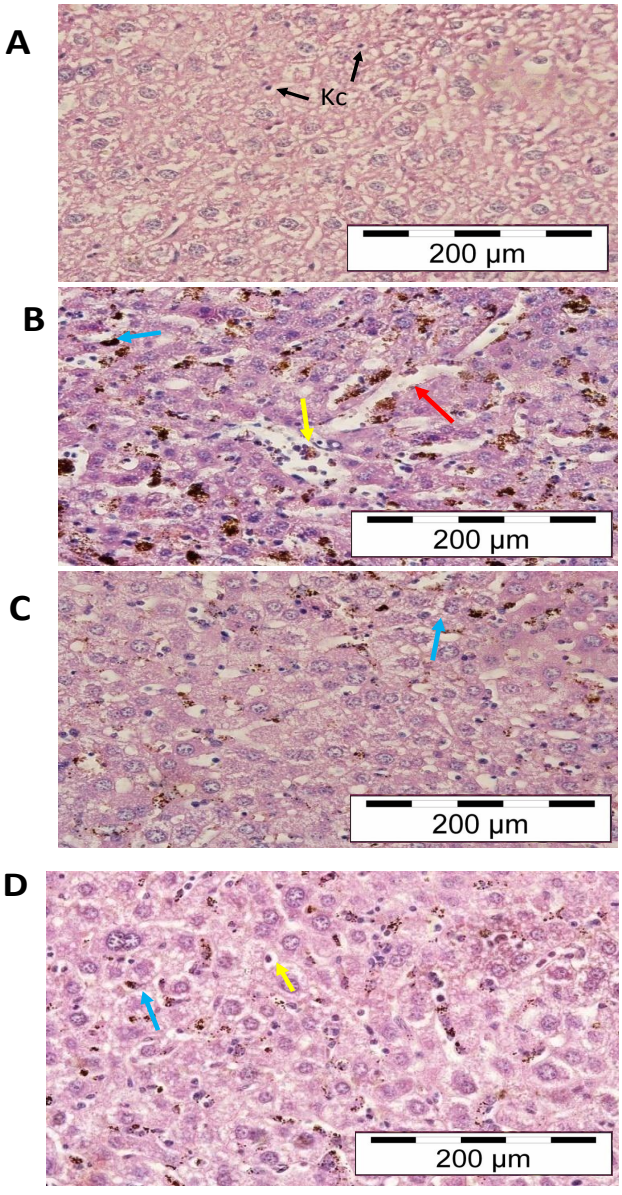


Figure 4: Light Micrographs of Liver Tissue of the PBS-treated Control Mice (A), PBS-treated- (B), rmIL-4-treated (C), rmIL-10-treated (D) and rmIL-13-treated (E) Malarial Mice. In PBS-treated malarial mice and rmIL-4, rmIL-13 treated mice, there were sequestration of pRBCs, pigment laden macrophage and lymphocytes in portal vein (black arrow). Infiltration of inflammatory cells (yellow arrow) was found in PBS and rmIL-10 treated mice. H & E stain, x400



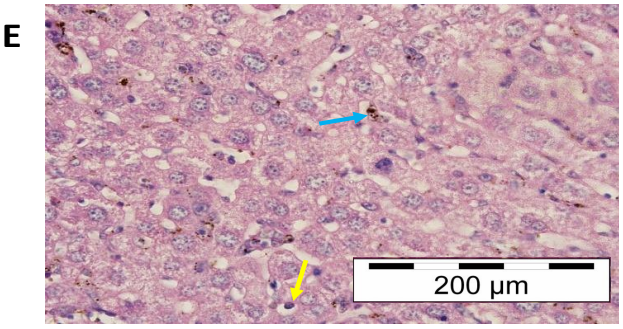


Figure 5: Light Micrographs of Liver Tissue of PBS-treated Control Mice (A), PBS-treated- (B), rmIL-4-treated (C), rmIL-10-treated (D), and rmIL-13-treated (E) Malarial Mice. Haemozoin pigmentation (blue arrow) and hypertrophy and hyperplasia of Kupffer cells (yellow arrow) were observed in all malarial mice. PBS-treated malarial mice showed significant dilatation of sinusoids (red arrow). Malarial mice receiving recombinant anti-inflammatory cytokines showed overall improvements on the above-mentioned pathological features. H & E stain, x400. Kc: Kupffer cells

Spleen

Splenic tissues of PBS-treated malarial mice exhibited loss of central germinal structure caused by expansion of red and white pulps (Figure 6B). Additionally, massive congestion of malarial pigments, lymphocytes and plasma cells in splenic cords and sinuses were observed in PBS-treated malarial mice (Figure 7B). PRBCs and macrophage engulfing elements were found to be sequestered along the splenic vessels, resulting in diffuse haemorrhage in malarial mice treated with PBS (Figure 7B). In contrast, malarial mice treated with rmIL-4, rmIL-10 and rmIL-13 had successfully retained central germinal structure along with more defined red and white pulps (Figure 6C, 6D and 6E). The degree of pigmentation and pRBCs sequestration were minimized with small amounts of the pigments and sequestered pRBCs presence around the splenic cord and vessels (Figure 7C, 7D and 7E). Diffused haemorrhagic feature was not detected in malarial mice treated with rmIL-4, rmIL-10 and rmIL-13 (Figure 7C, 7D and 7E).

Kidney

Enlargement of glomerulus with slight increment in the cellularity were observed in kidney tissue of PBS-treated malarial mice. Massive vacuolation was found within the glomerular tissue and kidney tubules suggesting the occurrence of glomerulonephritis and acute tubular necrosis (Figure 8B and 9B). The microvasculature was also sequestered with pRBCs, lymphocytes and pigment-laden macrophages (Figure 8B). The renal medulla of PBS-treated malarial mice showed a large amount of sequestered pRBCs along the interstitial blood vessels in association with deposition of pigment laden macrophages in interstitial tissue (Figure 9B). The renal tissues of mice receiving rmIL-4 and rmIL-13 therapies seemed devoid of

any significant pathology and appeared to be normal with little sequestration of pRBCs in the microvessels (Figure 8C and 8E, Figure 9C and 9E). Treatment of malarial mice with rmIL-10 significantly improved the appearance of tubular and glomerular tissue of malarial mice although the macrophage engulfing elements were still occasionally observed (Figure 8D and Figure 9D).

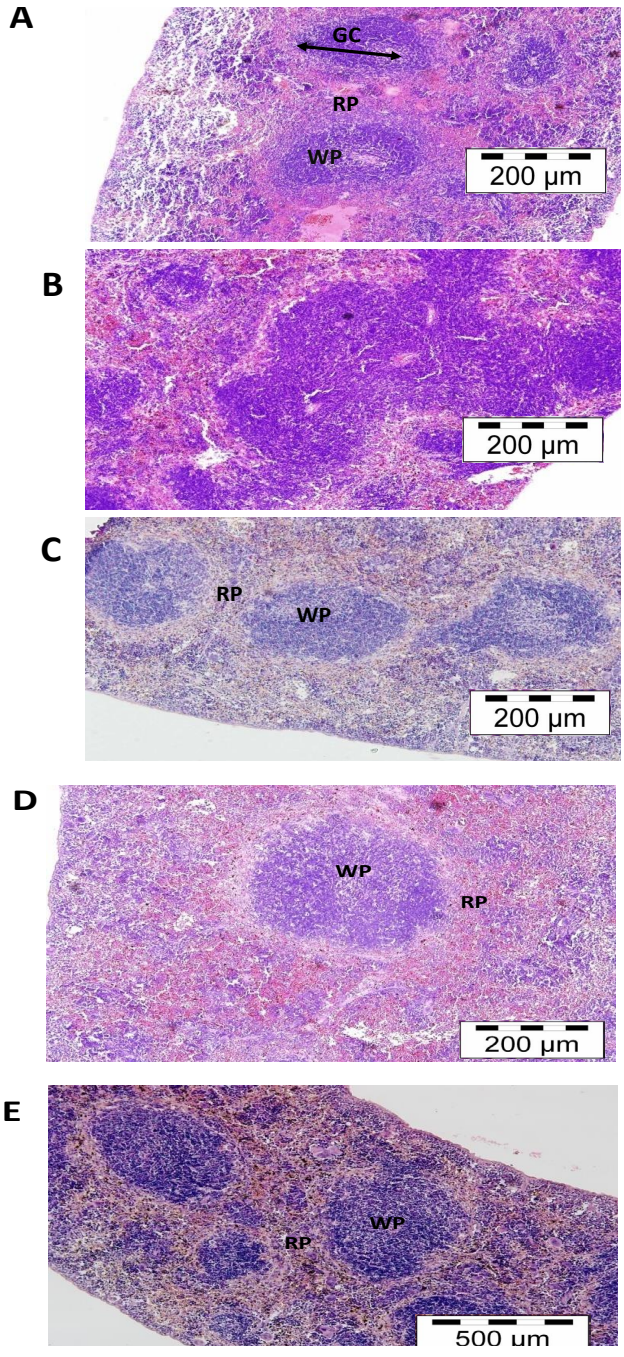


Figure 6: Light Micrographs of Splenic Tissue of PBS-treated Control Mice (A), PBS-treated- (B), rmIL-4-treated (C), rmIL-10-treated (D) and rmIL-13-treated (E) Malarial Mice. Enlarged red pulp (RP) and white pulp (WP) along with disappearance of germinal center was observed in (B). The architecture of WP and RP were preserved in rmIL-4, rmIL-10 and rmIL-13 treated mice. A, C, E – H & E stain, x100; B, D – H & E stain, x200. GC: germinal center

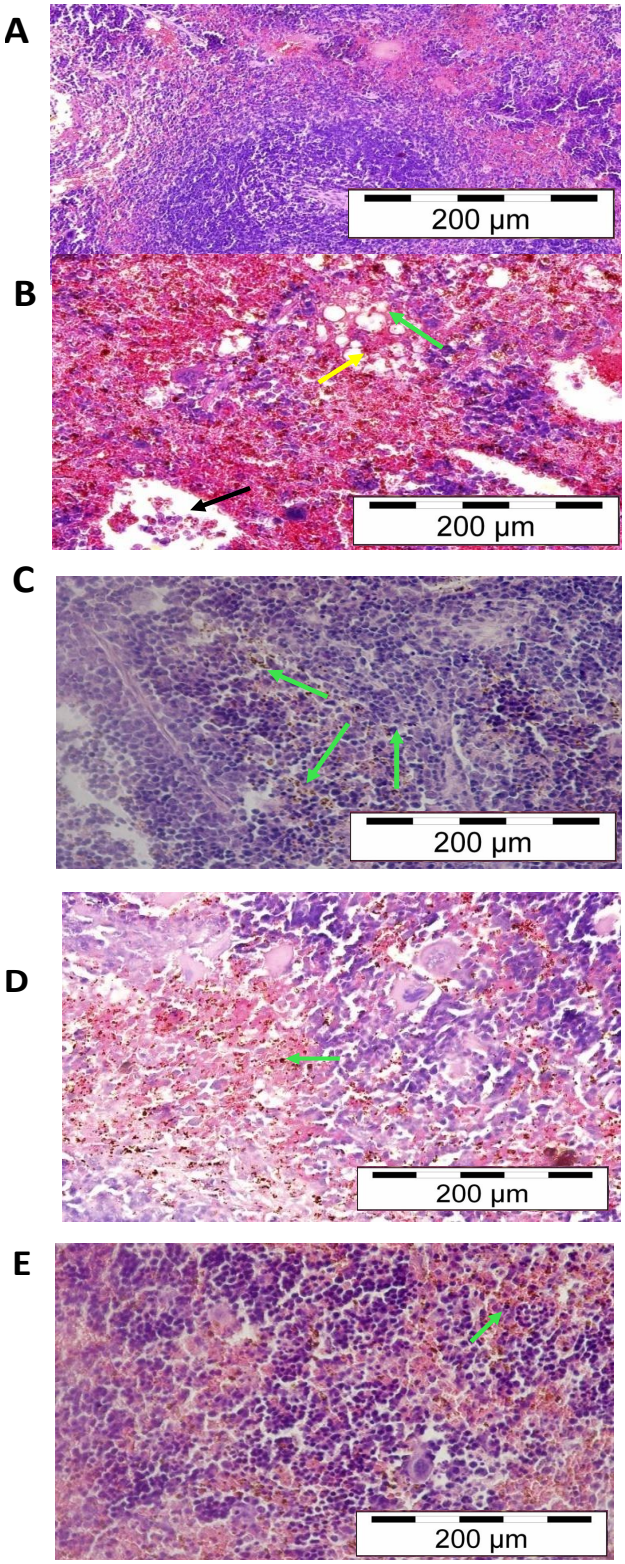


Figure 7: Light Micrographs of Splenic Tissue of PBS-treated Control Mice (A), PBS-treated- (B), rmIL-4-treated (C), rmIL-10-treated (D) and rmIL-13-treated (E) Malarial Mice. The pathologic pattern observed in PBS-treated malarial mice were pRBCs sequestration (black arrow) in vessels along with abundant pigment deposition in splenic sinuses and cords (green arrow). Diffuse haemorrhage (yellow arrow) was also found in the interstitial space of the tissue. Upon treatment with rmIL-4, rmIL-10 and rmIL-13, some haemozoin pigments were still deposited in sinuses and cords. H & E stain, x400

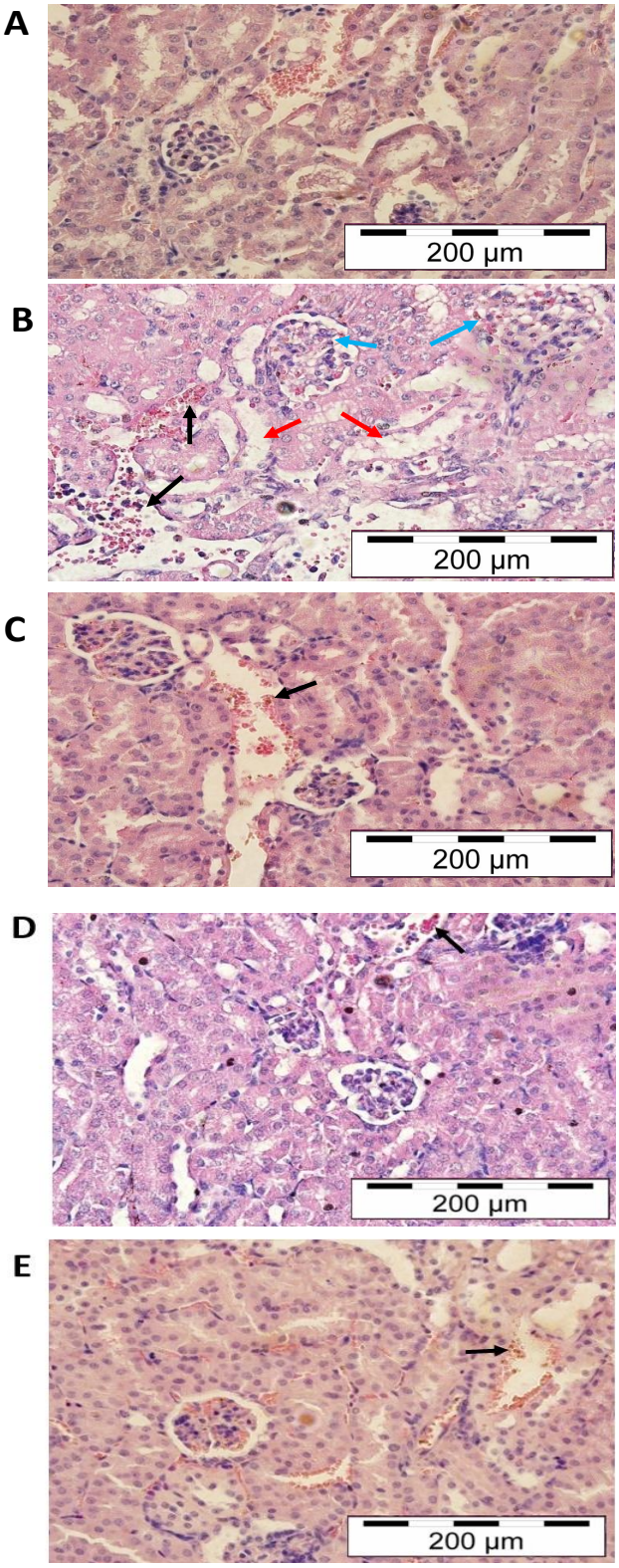


Figure 8: Light Micrographs of Kidney Tissue of PBS-treated Control Mice (A), PBS-treated- (B), rmIL-4-treated (C), rmIL-10-treated (D) and rmIL-13-treated (E) Malarial Mice. In PBS-treated malarial mice, features of enlarged glomerular (blue arrow), and tubular vacuolation (red arrow) was observed. Sequestration of pRBCs, macrophage engulfing elements and lymphocytes (black arrow) were less pronounced in C, D, E in association with normal appearance of kidney tissue as compared to B. H & E stain, x400

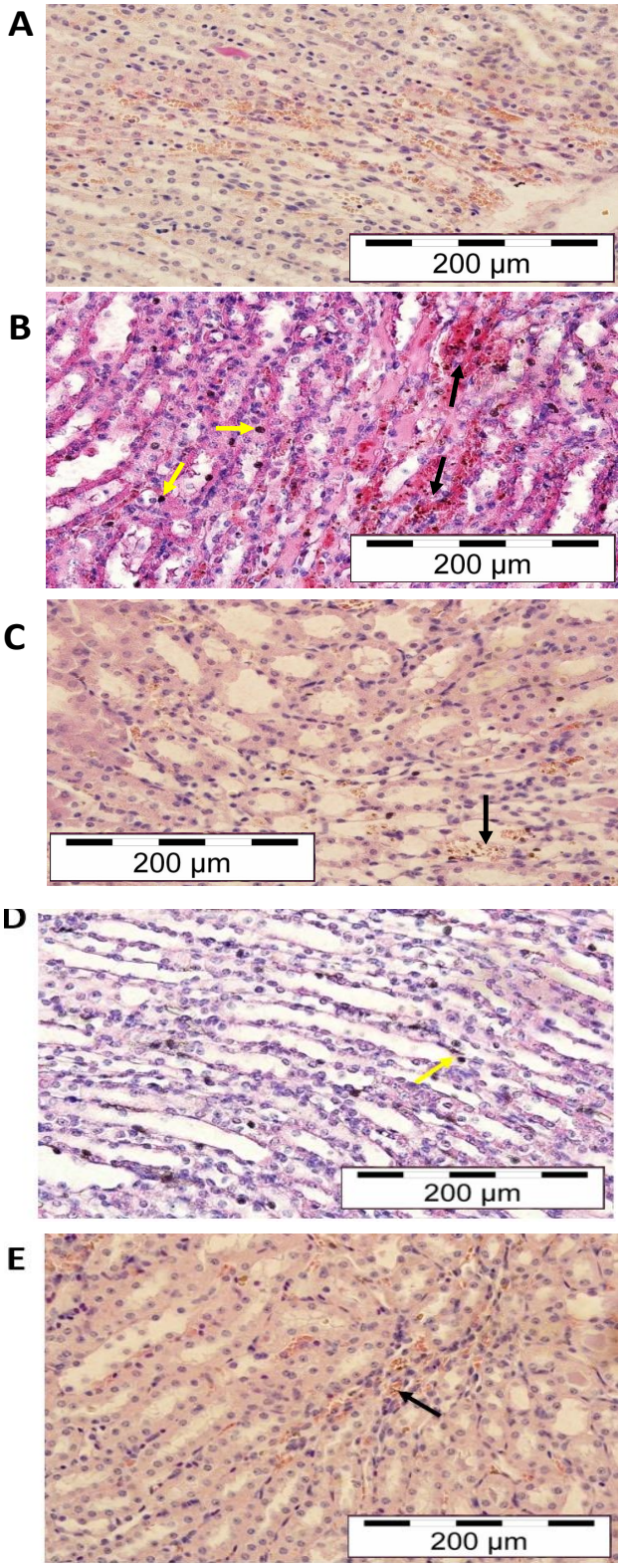
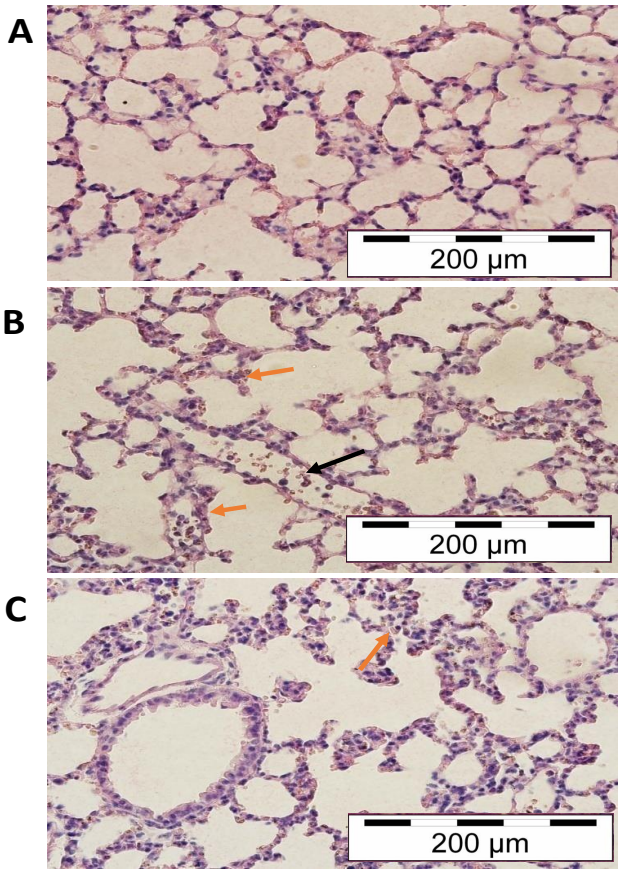


Figure 9: Light Micrographs of Kidney Tissue of PBS-treated Control Mice (A), PBS-treated- (B), rmIL-4-treated (C), rmIL-10-treated (D) and rmIL-13-treated (E) Malarial Mice. Sequestration of pRBCs and (black arrow) and deposition of pigment laden macrophage (yellow arrow) were observed in PBS-treated malarial mice in association of medullary hemorrhage. All malarial mice receiving anti-inflammatory cytokine related drugs showed very little sequestration and a normal medullary appearance. H & E stain, x400

Lungs

In lungs, abundance of hemozoins and pRBCs were found congested in the alveolar septa which subsequently leading to thickening of septal wall, an indicative of septal edema. The microvasculature was sequestered with substantial amounts of pRBCs. Macrophages with massive phagocytosed hemozoin pigments were frequently observed in the microvessels and alveolar septa (Figure 10B). The deposition of malarial pigments and sequestered pRBCs in the interalveolar spaces were minimized along with normal alveolar dispersion upon treatments of malarial mice with rmIL-4 and rmIL-13, (Figure 10C and 10E). Treatment of malarial mice with recombinant mouse IL-10, however, failed to clear the parasites from the microvessels and the alveolar regions appeared to be clustered (Figure 10D). A thick hyalinization of blood vessels was pronounced in PBS-treated malarial mice which were suspected to be a consequence of compromised alveolar-capillary barrier which could lead to diffuse damage in the alveoli (Figure 11B). Normal vascular appearances together with the absence of hyaline membrane on alveolar wall were observed in malarial mice treated with rmIL-4, rmIL-10 and rmIL-13 (Figure 11C, 11D and 11E).



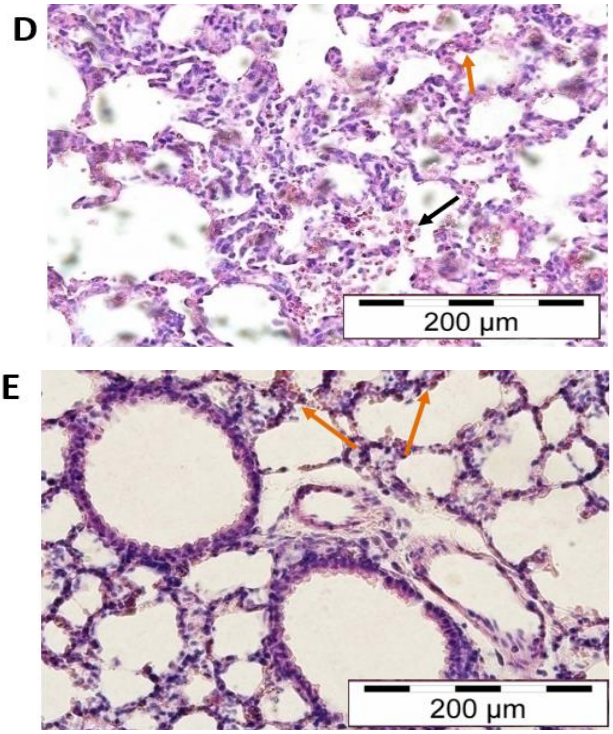
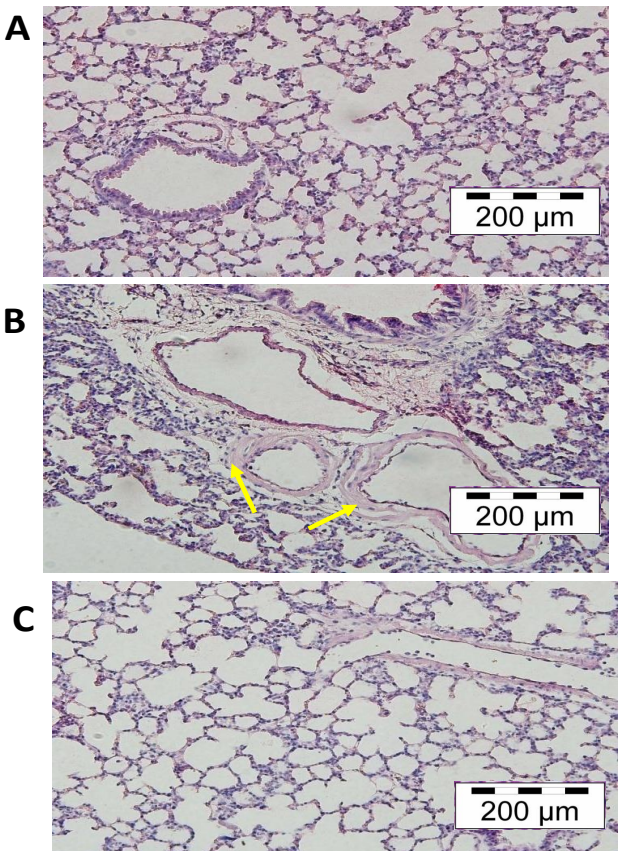


Figure 10: Light Micrographs of Lung Tissues of PBS-treated Control Mice (A), PBS-treated- (B), rmIL-4-treated (C), rmIL-10-treated (D), and rmIL-13-treated (E) Malarial Mice. PRBCs sequestration (black arrow) were found in microvessels of PBS-treated and rmIL-10 treated malarial mice. In all malarial mice, alveolar septa are thickened and congested with pRBCs and pigment laden macrophages (orange arrow). H & E stain, x400



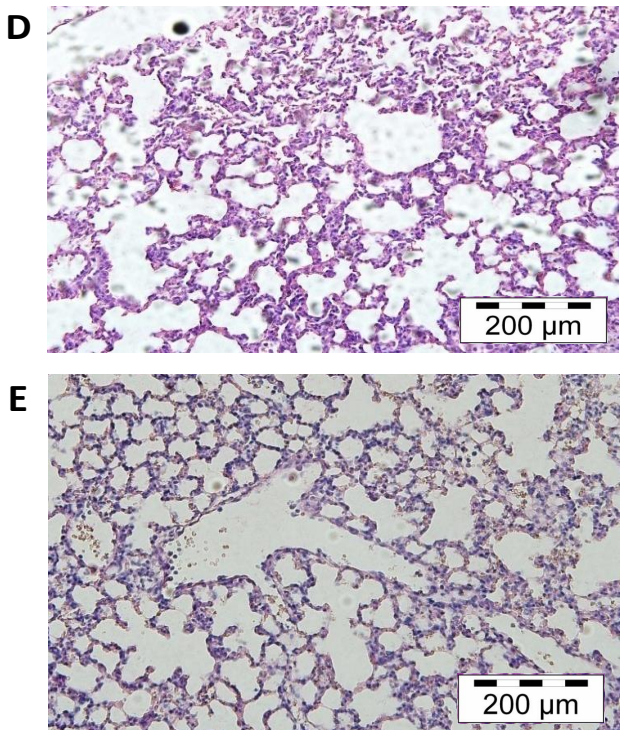


Figure 11: Light Micrographs of Lung Tissues in PBS-treated Control Mice (A), PBS-treated- (B), rmIL-4-treated (C), rmIL-10-treated (D), and rmIL-13-treated (E) Malarial Mice. A feature of hyaline membrane formation was found at the alveolar wall of PBS-treated malarial mice (yellow arrow). Normal alveolar appearances were observed in rmIL-4, rmIL-10 and rmIL-13 treated malarial mice. H & E stain, x200

Discussion

The favorable outcome of malaria infection is the clearance and elimination of parasitized cells without leaving severe pathologies. It has been well established that malaria pathogenesis is mediated by distorted inflammatory responses which could intensify the severity of the infection. The host genetic background and other environmental factors further modulate the innate and adaptive immunity to malaria. Anti-inflammatory cytokines, one of the potent antimalarial regulators have been recognized as significant modifiers of effector immune responses through neutralization of pro-inflammatory responses (9, 24, 25). Hence, in this study, the effect of recombinant mouse (rm) IL-10, rmIL-4 and rmIL-13 in malarial mice were investigated to determine whether the enhancement of these anti-inflammatory cytokines could ameliorate the severe pathology contributed by malaria antigens using experimental PbA-infected murine model.

In this study, peak parasitemia level was observed in malarial mice at day-6 post infection. The high parasitemia level is accompanied with high mortality rate in malarial mice at day-6 post infection. Conversely, the susceptibility towards *P. berghei* ANKA (PbA) infection was reduced in malarial mice receiving rmIL-4, rmIL-10 and rmIL-13, evident by prolonged lifespan in malarial groups receiving those treatments compared to malarial mice receiving PBS. Also, the parasitemia levels in malarial groups treated with rmIL-10 and rmIL-13 were significantly reduced at late stage of infection with delayed mortality were recorded. We postulate that high levels of malaria parasitemia accompanied with excessive inflammatory responses contributed to the high mortality rate in malarial mice in this study. This is supported by our previous work where high levels of TNF-α and IL-1α cytokines, but low level of IL-4 cytokine was produced during peak parasitemia in a severe murine model of malaria infection (26). Higher levels of IL-10 and IL-13 cytokine expressions were also observed in that particular study (26). On the other side, significant reduction in malaria parasitemia was not recorded in malarial mice receiving recombinant IL-4. This could be caused by inadequate supply of exogeneous IL-4 despite recombinant IL-4 treatment was applied to compensate the low level of IL-4 production during severe malaria infection, which might affect the parasite clearance and mediation of inflammation process. In turn, high levels of IL-10 and IL-13 cytokine expressed during severe malaria infection (26) along with additional exogenous supply of IL-10 and IL-13 cytokines successfully suppressed malaria parasitemia development and prolonged the survival of malaria mice.

The precise mechanisms for the reduced susceptibility of malarial mice receiving rmIL-4, rmIL-10 and rmIL-13 to malaria infection remain elusive. The reduction could be due to stimulation of signal transducer and activator of transcription 6 (Stat6). Stat6 is responsible for exerting biological activities induced by IL-4 and IL-13, including the suppression of Th1 cells and proinflammatory cytokines production. The suppression of Th1-related cytokines mediated by Stat6 is likely occurs through competitively binding with DNA of NF-κB (27, 28) and/or by enhancing the expression of anti-inflammatory mediators such as IL-10 (29). IL-10 is a potent anti-inflammatory cytokine involved in malaria pathogenesis. A few studies stated that the severity of malaria infection was exaggerated in IL-10 knockout mice (IL-$10^{-/-}$) infected with *P. chabaudi* in comparison with wild-type (30, 31). Also, IL-10 is often associated with increasing resistance to infection or reduced disease severity in malaria and other pathogens such as *Toxoplasma gondii* and *Helicobacter hepaticus*. This suggests that IL-10 plays a central role in mitigating the inflammatory responses and provoking a strong Th2-like response (30). Thus, it is not surprising that treatments with Th2-mediators such as IL-10 and IL-13 cytokines in malaria mice could both alleviate the parasitemia levels and survivals of malarial mice. Maintaining the equilibrium between tissue protective roles of IL-10 and IL-13 cytokines and their suppressive effects on anti-parasitic immune responses are therefore crucial for designation of immunotherapy that exploits IL-10 and IL-13.

The onset of experimental cerebral malaria (ECM) is typically manifested by the sequestration of malaria parasites in various organs such as brain, liver, kidney, lung and spleen (32). The disease manifestations in

brain during malaria infection are often associated with several pathological events such as microvessel damages and haemorrhage in brain microvascular endothelial cells, enlargement of perivascular space, increased in vascular permeability and the presence of necrotic and pyknotic neurons accompanied with massive influx of pro-inflammatory cytokines, which further exacerbated the brain pathology during malaria infection (33). Our study showed that malarial mice receiving rmIL-4, rmIL-10 and rmIL-13 therapies have significantly improved the brain pathology, for example, sequestration of pRBCs in the microvascular endothelial cell was absent along with normal appearance of neurons. This explains that enriching anti-inflammatory cytokines in early stage of infection could effectively prevent the cerebral pathology caused by the Th1 response (34, 35). Apart from that, hemozoin depositions and sequestrated pRBCs conditions in the liver of malarial mice were alleviated upon treatments with rmIL-4, rmIL-10 and rmIL-13. The presence of hypertrophied Kupffer cells which are frequently observed during malaria infection, were also lessen upon treatments with anti-inflammatory cytokines, indicating a counter regulation of immune response that reduces inflammation in liver (36). Additionally, previous study reported that administration of exogenous anti-inflammatory cytokines improved the hepatic blood flow by suppressing the expression of pro-inflammatory cytokines and chemoattractant CXCL10 (37).

Spleen is a secondary lymphoid organ that is closely involved in host defense against malaria infection. Enlargement of spleen with dark coloration was found in malarial mice. Also, disorganization of germinal center architecture was apparent as a result of hyperplasia in red and white pulps. Splenic vessels, cords and sinuses were sequestered with pRBCs, uninfected RBCs, mononuclear cells and pigment-laden macrophages. The anti-inflammatory cytokines (IL-4, IL-10 and IL-13) appeared to protect the malarial mice by rectifying the pathological changes in the malarial spleen. Administration of rmIL-4, rmIL-10 and rmIL-13 in malarial mice minimized the loss of spleen architectural by retaining a distinct red and white pulp formation as well as the germinal center, therefore allowing for B cell differentiation and centrocyte transformation into IgG-producing plasma cells that responsible for induction of effective immunological memory response (38, 39). Meanwhile, hemozoin pigments remain to be deposited in sinuses and cords of malarial mice receiving rmIL-4, rmIL-10 and rmIL-13 therapies. Parasitemia level is a weak predictor to measure the extent of hemozoin spread in spleen. It is suggested that the reduced parasitemia level in malarial mice treated with rmIL-4, rmIL-10 and rmIL-13 was due to splenic response that promoted filtration capacity for antigen trapping (40). During murine malaria infection, the splenic reticuloendothelial system was activated to entrap pRBCs and other senescent erythrocytes. These reticular cells might act synergistically with follicular dendritic cells to enhance the removal of intraerythrocytic parasites through antibody-independent killing mechanisms and returned the intact RBCs to the splenic circulation (41, 42).

In kidney, glomerular and medullary tissue of malarial mice treated with rmIL-4, rmIL-10 and rmIL-13 demonstrated minor morphological abnormalities with lesser pRBCs were sequestered in interstitia compared to severe pathological changes observed in PBS-treated malarial mice. Published data indicated that the fluctuating productions of pro- and anti-inflammatory cytokines during malaria infection are associated with acute renal inflammation and disorders in rodents (43, 44). Upon stimulation of malarial antigens and toxins, renal endothelial cells and glomerular intrinsic cells facilitate the synthesis of pro-inflammatory cytokines TNF-α, IL-1α and hence upregulation of cell adhesion molecules that responsible for murine malarial glomerulonephritis. Increased staining of pro-inflammatory cytokines and reduced staining of anti-inflammatory cytokines revealed by previous immunohistochemistry analysis also suggested its causal contribution towards malarial tubulointerstitial nephritis (45). The absence of glomerular hypercellularity and tubular vacuolation in malarial mice receiving rmIL-4, rmIL-10 and rmIL-13 therapies could be due to the suppressive effects exerted on Th1 cytokines by macrophages exemplified by decreased in the deposition of sequestered pRBCs and pigmented macrophages. The findings in this study showed that administration of anti-inflammatory cytokine related drugs could alleviate acute renal disorders and limit the excessive effector response of pro-inflammatory mediators in diseased kidney.

In lungs, hyaline membrane was absent in the alveolar wall of malarial mice treated with rmIL-4, rmIL-10 and rmIL-13. Sequestration of pRBCs and pigment laden macrophages without marked thickening of alveolar septa were less observed in the lungs of malarial mice receiving anti-inflammatory cytokines related drugs. It is likely that parasite deposition was proportionate to the relative parasitemia recorded during therapy as lung is one of the organs with major *P. berghei* sequestration (46). Histological analysis confirmed that malarial pigments were localized in phagocytes and pRBCs, reflecting the increased activity of pulmonary phagocytosis. The pathogenesis of lung injury could be caused by the enhancement of pro-inflammatory signals expression by activated macrophages (47, 48). Additionally, pulmonary inflammation characterized by substantially high levels of TNF-α and IL-1 might to accumulation of hemozoins in the microvessels and subsequent disruption of alveolar-capillary functions (49, 50). The low burden of sequestered pRBCs observed in the lung tissues of malarial mice receiving rmIL-4, rmIL-10 and rmIL-13 could be explained by the protection conferred by monocytes and macrophages through antibody dependent cellular inhibition and the balance between pro- and anti-inflammatory responses during malaria infection. Altogether, the results showed that the administration of rmIL-4, rmIL-10 and rmIL-13 significantly improved the pathological lesions in the PbA-infected lungs.

Conclusion

In conclusion, our study has several limitations. We did not detect the expressions of IL-4, IL-10 and IL-13 cytokines endogenously before and after treatments in malarial mice under different treatment groups which could inform us in more details on the involvement of these cytokines in malaria pathogenesis and to further explain the findings obtained in this study including suppression of malaria parasitemia development, prolonged survival and alleviation of histopathological conditions in major affected organs were because of direct effects of these recombinant anti-inflammatory cytokines or not. Also, both qualitative and quantitative assessments including histological scoring and immunohistochemical staining were not performed in this study. These could provide us a clearer picture to understand to what extent the modulating effects of IL-4, IL-10 and IL-13 mediators on the histopathological changes of major affected organs during malaria infection. The study was also limited using single therapeutic dosage and a short treatment period. Optimal dosage and adjustment of the length of treatments might improve the integrity of the findings. Nonetheless, this preliminary study revealed the potential of using Th2 mediators such as IL-4, IL-10 and IL-13 cytokines for immunotherapeutic applications during malaria infection. Further investigations are warranted to unravel the precise functional roles of these mediators and the mechanisms involved in malaria infection.

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Competing Interests

The authors declare that they have no competing interests.

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